



IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

RECEIVED  
JUN 14 2002  
TECH CENTER 1600/3000

Applicant : Cindie M. Luhman

Serial No. : 09/239,873

Filed : January 29, 1999

For : METHOD AND COMPOSITION FOR  
ENHANCING MILK COMPONENT  
CONCENTRATIONS

Group Art Unit: 1653

Examiner: N. Levy

Docket No. : LL11.12-0040

**DECLARATION UNDER 35 U.S.C. §132**

#19  
Aftermon  
6/20/02

Commissioner of Patents and Trademarks  
Washington, D.C. 20231

**SENT VIA EXPRESS MAIL**  
**Express Mail No.: EV030213287US**

Sir:

I, Paul A. Porter, of 1025 190<sup>th</sup> Street, Webster City, Iowa, hereby declare as follows:

1. I am currently the Dairy Research Manager of the Land O' Lakes Research Farm in Webster City, Iowa.
2. I obtained a Bachelor of Science (B.S.) degree in Chemistry from Wittenberg University of Springfield, Ohio in 1981.
3. I obtained a Masters of Science (M.S.) in Animal Science from Cornell University in Ithaca, New York in 1984.
4. I obtained a Doctorate of Philosophy (Ph.D.) in Animal Science, with an emphasis in Dairy Nutrition, from Cornell University in Ithaca, New York in 1987.

**Declaration of Paul A. Porter under 35 U.S.C. §132**

2-

5. Since completing my Doctorate of Philosophy degree in 1987, I have continuously been employed in positions directly relating to dairy herd management and nutrition.
6. From 1987 to 1989, I was an Assistant Professor in the Department of Animal Science at Oregon State University in Corvallis, Oregon, where I conducted applied dairy nutrition research; taught courses in ration balancing, dairy herd management, and advanced dairy herd management; and served as a liaison to the Oregon State Dairy Industry.
7. From 1989 to 1992, I was the Dairy Program Manager for Countrymark Co-op of Delaware, Ohio, where I provided technical assistance, troubleshooting expertise, product support, and training to local dairy cooperatives, sales staff of the local dairy cooperatives, and dairy farm clients of the local dairy cooperatives.
8. From 1992 to 1994, I provided dairy sales and dairy consulting for Young's Livestock Nutritional Services of Canastota, New York, where I analyzed dairy production records, consulted on dairy production issues, and designed customized dairy herd feeding programs for dairy farm clients.
9. From 1994 to 2000, I was Dairy Nutritionist and Field Technical Services Manager for Land O' Lakes Dairy Feed of Sun Prairie, Wisconsin, where I provided technical assistance, Troubleshooting services, product support, and training to local dairy cooperatives, sales staff of the local dairy cooperatives, and dairy farm clients of the local dairy cooperatives.

**Declaration of Paul A. Porter under 35 U.S.C. §132**

3-

10. From 2000 to the present, I have been Dairy Research Manager for Land O' Lakes Research Farm of Webster City, Iowa, where I am responsible for design, implementation, and summarizing dairy research trials for the farm's dairy herd that includes 240 cows. Additionally, as Dairy Research Manager, I assist in new feed product development and coordinate with the Feed Division of Land O' Lakes, Inc. regarding feeding trial results. Finally, as Dairy Research Manager, I am responsible for my group's annual budget of approximately \$700,000.00.
11. A brief resume of my educational career and my professional career from 1981 to the present is attached hereto as Exhibit A.
12. I am experienced in dairy herd feeding experiments and trials, including both *in vitro* and *in vitro* approaches, due my education and work experience relating to dairy nutrition issues over the past 18 years.
13. The term *in vitro* describes "a biological reaction which can be performed outside the living organism in the laboratory; as, in a test tube or petri dish, on a microscope slide, etc.", whereas the term *in vivo* describes "a reaction which takes place within the living organism," according to Grant, Roger and Grant, Claire, Grant & Hackh's Chemical Dictionary, page 307 (5<sup>th</sup> edition McGraw-Hill Book Company), which is attached to this Declaration as Exhibit B.
14. *In vivo* experiments exploring the function of microorganisms in the rumen of living animals and *in vitro* experiments exploring the function of rumen microorganisms in a laboratory and isolated from the rumen of the animal are described in Hobson,

**Declaration of Paul A. Porter under 35 U.S.C. §132**

4-

P.N. The Rumen Microbial Ecosystem, pages 461-463 (1998, 1<sup>st</sup> Edition, Elsevier Science Publishers Ltd.), which is attached to this Declaration as Exhibit C. Exhibit C further indicates that *in vitro* results are not always reproducible under *in vivo* conditions in the rumen of a ruminant.

15. Similarly, Hobson, P.N. and Stewart C.S., The Rumen Microbial Ecosystem, pages 661-662 (1997, 2<sup>nd</sup> Edition, Chapman and Hall), which is attached to this Declaration as Exhibit D, states that *in vitro* conditions “might reproduce metabolic pathways of rumen microorganisms” that occur under *in vivo* conditions, but do not always or necessarily reproduce *in vivo* results.
16. As support for the facts provided in Paragraph 15 of this Declaration, Exhibit D states that *in vitro* experiments “could not always” quantitatively reproduce metabolic reactions of rumen microorganisms observed under *in vivo* conditions and further states: “The only ‘container’ that reproduces the rumen is the rumen.”
17. Exhibit C posits some reasons why rumen microorganism reactions under *in vitro* conditions do not always reproduce rumen microorganism reaction results observed under *in vivo* conditions. For example, Exhibit C states:

The fact that the reactions *in vitro* do not reproduce those *in vivo* does not necessarily mean that the test organism has no place in the rumen system; it may be that it’s growth conditions in the rumen have not been properly reproduced in the laboratory, or it may mean that it’s growth in the rumen is overshadowed by some unisolated organism.”
18. Indeed, as observed in Hobson, P.N., and Stewart, C.S., The Rumen Microbial Ecosystem, page 496 (1997, 2<sup>nd</sup> Edition, Chapman and Hall), which is attached to this

**Declaration of Paul A. Porter under 35 U.S.C. §132**

5--

Declaration as Exhibit E, the rumen is a complex system with many interrelated sub-systems:

The rumen has a complex structure and the contents are heterogeneous, consisting primarily of a microbial suspension in free liquid, a solid mass of digesta, and a gas phase. Each of these entities is complex; the properties of microorganisms in suspension deep in the rumen might be different from those of microorganisms close to the rumen wall (Cheng and Costerton, 1980.) The solid mass of digesta is certainly heterogeneous, and even the gas phase is complex, consisting of a large gas space (gas-cap), smaller pockets of gas entrapped within the solid mass, and the dissolved gas. Many properties of individual parts of the microbial system of the rumen have been investigated, but often the investigations were governed by the ease of experimentation rather than the importance of the given component. This is why the complex, and relatively inaccessible, semi-solid mass of digesta has received so little attention. In addition, worthwhile investigations were hampered by a lack of simple conceptual basis for dealing with such a complex system.

19. Further factual explanation about the inconsistencies and variations observed between *in vitro* results versus *in vivo* results in relation to rumen metabolism, as mentioned in Paragraphs 14 to 17 above, is provided in Hobson, P.N. and Stewart, C.S., The Rumen Microbial Ecosystem, page 518 (1997, 2<sup>nd</sup> Edition, Chapman and Hall), attached to this Declaration as Exhibit F, which states:

The microbial system of the rumen is not a simple fermentation vat filled with randomly distributed mixtures of microorganisms. It is a highly structured and functionally compartmented system, on a par with any of the organs of the advanced multicellular organism. Significant progress in the field of rumen metabolism is contingent on appreciation of the rumen and its microbial ecosystem in this manner.

**Declaration of Paul A. Porter under 35 U.S.C. §132**

6--

20. Continuing, Hobson, P.N., The Rumen Microbial Ecosystem, page 428 (1988, 1<sup>st</sup> Edition, Elsevier Science Publisher's Ltd.), which is attached to this Declaration as Exhibit G, explains that complex "interrelations within the system of rumen metabolism," such as "microbial growth yield" issues and "digesta kinetics" issues make "the study of rumen optimization a very laborious task" and suggests that this complexity of interrelations within the rumen metabolism system is the primary reason for variability and contradictory results between *in vitro* and *in vivo* systems regarding rumen metabolism:

The complexity of the rumen and the ruminant systems to be manipulated is the main reason for variability and contradiction in experimental results.

21. As one example of the variability that may be expected between *in vitro* and *in vivo* experimental results relating to rumen metabolism, Hobson, P.N. and Stewart C.S., The Rumen Microbial Ecosystem, pages 586-587 (1997, 2<sup>nd</sup> Edition, Chapman and Hall), which is attached to this Declaration as Exhibit H, states that fiber digestibility upon isoacid supplementation showed a positive response during under *in vitro* conditions, but showed only a minimal or insignificant response under *in vivo* conditions during a rumen metabolism study.

22. Next, Hobson, P.N., The Rumen Microbial Ecosystem, pages 140-141 (1988, 1<sup>st</sup> Edition, Elsevier Science Publishers Ltd.), which is attached to this Declaration as Exhibit I, observed that added haem created a zoosporogenesis under *in vivo* rumen conditions, "but this could not be satisfactorily repeated *in vitro* with pure cultures, suggesting that the control of zoosporogenesis and zoospore really may be more complex than suggested by the work *in vivo*." (Emphasis added.)

**Declaration of Paul A. Porter under 35 U.S.C. §132**

7-

23. Finally, even where rumen system complexity is not a factor, Santoro, Luiz G., Grant, George, and Pusztai, A. Arpad, Differences in the Degradation *in Vivo* and *in Vitro* of Phaseolin, the Major Storage Protein of *Phaseolus Vulgaris* Seeds, pages 612-613, Biological Society Transactions - 625<sup>th</sup> Meeting (London 1998), which is attached to this Declaration as Exhibit J, demonstrates that large quantitative variations between *in vivo* and *in vitro* nevertheless can occur. Specifically, in the study that was the subject of Exhibit H, digestive degradation of phaseolin (glycoprotein II, G-II) under *in vivo* conditions was about 74%, while the extent of digestive degradation of phaseolin under *in vitro* conditions in hamsters was only about 2%, which is vastly different from the results obtained under *in vivo* conditions.
24. Thus, the collective teaching of the factual observations and statements in Paragraphs 14 to 22 above is that *in vitro* simulations of rumen metabolism, at least from a quantitative perspective, and possibly from other perspectives, are not a reliable predictor of *in vivo* rumen metabolism results, but are instead speculative. Furthermore, the factual observations and statements of Paragraphs 14 to 22 above demonstrates that, for the same substrate, widely different quantitative results may be observed when comparing *in vitro* rumen metabolism experimental results to actual *in vivo* rumen metabolism results.
25. I am familiar with the disclosure of U.S. Patent No. 4,127,676 to Merensalmi (the "Merensalmi Patent"), which is attached to this Declaration as Exhibit K.

**Declaration of Paul A. Porter under 35 U.S.C. §132**

8–

26. In Example 1, the Merensalmi Patent presents *in vitro* testing results regarding sugar alcohol preservation “in rumen fluid.” (Column 2, lines 59-63, of the Merensalmi Patent of Exhibit K).
27. The Merensalmi Patent does not provide any details whatsoever regarding the source, composition, handling, or preservation of the rumen fluid used in the *in vitro* testing referenced in paragraph 26 above and does not provide any details whatsoever about the experimental protocol or procedure for the Example 1 *in vitro* testing of sugar alcohol preservation in “rumen fluid.”
28. Nonetheless, despite providing no evidence about the *in vitro* testing protocol or procedure and despite providing no evidence about the source, composition, handling, or preservation of the rumen fluid, the Merensalmi Patent nevertheless alleges that the *in vitro* testing results of Example 1 conclusively establish that similar sugar alcohol degradation rates to those presented in Example 1 *would* occur under *in vivo* conditions in the rumen of a living ruminant:
- The present invention, however, is based upon the prior unknown fact that sugar alcohols remain intact also under the conditions in the rumen sufficiently long without breaking down, which appears from the [in vitro] test results presented in Example 1.
- (Column 2, lines 53-57, of the Merensalmi Patent of Exhibit K; emphasis added).
29. Furthermore, the Merensalmi patent provides no evidence or reasoning whatsoever in support of the statement recited in Paragraph 28 above alleging that the *in vitro* testing results of Example 1 conclusively establish that similar sugar alcohol



**Declaration of Paul A. Porter under 35 U.S.C. §132**

9–

degradation rates to those presented in Example 1 *would* occur under *in vivo* conditions in the rumen of a living ruminant.

30. Based upon the collective teaching of the factual observations and statements in Paragraphs 14 to 22 above about *in vitro* simulations of rumen metabolism, at least from a quantitative perspective, not being a reliable predictor of *in vivo* rumen metabolism results, and based upon my factual observations in Paragraph 28 and 29 above, the allegation recited from the Merensalmi patent in Paragraph 28 above is simply an unsupported and inconclusive allegation that does not actually disclose, or support a conclusion, that similar sugar alcohol degradation rates to those presented in Example 1 *would* occur under *in vivo* conditions in the rumen of a living ruminant. Instead, the factual observations and statements of Paragraphs 14 to 22 above demonstrate that, for the same substrate, such as the individual sugar alcohols of Merensalmi Example 1, widely different quantitative results may be observed when comparing *in vitro* rumen metabolism experimental results to actual *in vivo* rumen metabolism results.

31. Building upon my factual observations of Paragraph 30, the Merensalmi Patent approach of equating the *in vitro* sugar alcohol degradation results of Merensalmi Example 1 to real life *in vivo* sugar alcohol degradation results that allegedly *would* be obtained under *in vivo* conditions using the complex rumen function of a live ruminant is mere speculation, since “The only ‘container’ that reproduces the rumen is the rumen.” (See Exhibit D).

**Declaration of Paul A. Porter under 35 U.S.C. §132**

10—

32. Indeed, based upon my factual observations and factual conclusions referenced in Paragraphs 27 and 29 to 31 above, the Merensalmi tactic of equating the *in vitro* results from Merensalmi Example 1 to results that *would allegedly* be obtained under *in vivo* conditions is speculative at best and does not in fact disclose, establish, or prove sugar alcohol degradation rates that would be experienced under *in vivo* conditions.
33. Based on my factual observations of paragraph 32 above, it follows that the tabular *in vitro* results that extend from column 2, line 65, through column 3, line 5, of the Merensalmi Patent pertain purely to the results of the Merensalmi *in vitro* testing under unspecified conditions and an unspecified testing protocol and do not establish, prove, or disclose anything about sugar alcohol degradation in the rumen of a living ruminant under *in vivo* conditions.
34. Example 2, which extends from line 12 to line 38 in column 3 of the Merensalmi Patent, provides additional test results for a sugar alcohol mixture, as opposed to the test results provided in Example 1 of the Merensalmi Patent for individual sugar alcohols.
35. Example 2, like Example 1, refers to testing “in the rumen fluid,” as opposed to *in vivo* testing in the rumen of a live ruminant, and therefore, like the test results of Example 1, merely amounts to *in vitro* experimentation about the sugar alcohol mixture degradation characteristics in “the rumen fluid.”

**Declaration of Paul A. Porter under 35 U.S.C. §132**

11-

36. The Merensalmi Patent does not provide any details whatsoever regarding the source, composition, handling, or preservation of the rumen fluid used in the *in vitro* testing referenced in paragraphs 34 and 35 above and does not provide any details whatsoever about the experimental protocol or procedure for the Example 2 *in vitro* testing of the sugar alcohol mixture preservation in "rumen fluid."

37. Furthermore, the Merensalmi patent provides no evidence or reasoning whatsoever that would support an allegation that the *in vitro* testing results of Example 2 conclusively establish that similar sugar alcohol degradation rates to those presented in Example 2 *would* occur under *in vivo* conditions in the rumen of a living ruminant.

38. Despite the complete lack of protocol, procedure, reasoning and evidence, Merensalmi Example 2 as with Example 1, alleges that the results of the Example 2 *in vitro* testing equate to results that *would* be obtained if the sugar alcohol mixture were instead subjected to *in vivo* testing in the rumen of a living ruminant:

As the flow of the fluid in the rumen is only a few hours, the sugar alcohol reaches the latter stomach compartments before any essential degradation in the rumen can occur.

(Column 3, lines 30-33, of the Merensalmi Patent of Exhibit K).

39. Nonetheless, despite this Merensalmi allegation that is recited in Paragraph 38 above, the results of the *in vitro* testing of Example 2, for reasons analogous to those provided in Paragraphs 30 to 32 above with regard to Merensalmi Example 1, do not establish, prove, or disclose sugar alcohol degradation rates under *in vivo* conditions in the rumen of a living ruminant and do not establish, prove, or disclose sugar alcohol degradation rates that *would* necessarily be expected upon replacing the *in*

**Declaration of Paul A. Porter under 35 U.S.C. §132**

12-

*vitro* procedure of Example 2 with a true *in vivo* procedure in the rumen of a living ruminant.

40. Instead, for reasons analogous to those provided in Paragraphs 30 to 32 above with regard to Merensalmi Example 1, the attempt of Merensalmi Example 2 to equate the *in vitro* sugar alcohol degradation rate results to *in vivo* results is purely speculative and without any evidence or basis in fact.

41. Finally, the last sentence in Example 4 of the Merensalmi Patent states:

The major part of the sugar alcohol mixture however passes through the rumen without breaking down.

(Column 4, lines 45-47, of the Merensalmi Patent of Exhibit K).

42. However, the only basis for this statement of Merensalmi Example 4 that is recited in Paragraph 38 above, are the erroneous and unsupported allegations of the Merensalmi patent regarding Examples 1 and 2 that are mentioned in Paragraphs 28 and 38 above

43. Consequently, for reasons analogous to those provided in Paragraphs 30 to 33 and 39 to 40 above, there is no evidence or factual basis in support of the Merensalmi Example 4 statement that is recited in Paragraph 43 above; consequently, for reasons analogous to those provided in Paragraphs 30 to 33 and 39 to 40 above, the statement recited in Paragraph 43 above regarding Merensalmi Example 4, like the prior Merensalmi statements equating the *in vitro* results of Examples 1 and 2 to *in vivo* results, is purely speculative and without any basis in fact.

First Named Inventor: Cindie M. Luhman

Application No.: 09/239,873

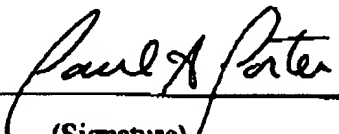
**Declaration of Paul A. Porter under 35 U.S.C. §132**

13-

44. Ultimately, considering the factual statements and factual observations provided in Paragraphs 26 to 43, the Merensalmi patent only provides details about *in vitro* sugar alcohol degradation testing under unspecified conditions and an unspecified testing protocol and does not establish, prove, or disclose anything about sugar alcohol degradation in the rumen of a living ruminant under *in vivo* conditions.

I declare that all statements made herein that are of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of this application or any patent issuing thereon.

Inventor: Paul A. Porter  
(Printed Name)

Inventor:   
(Signature)

Date 6/3/02



Applicant : Cindie M. Luhman

Serial No. : 09/239,873

Filed : January 29, 1999

For : METHOD AND COMPOSITION FOR  
ENHANCING MILK COMPONENT  
CONCENTRATIONS

Docket No. : LL11.12-0040

Group Art Unit: 1616

Examiner: N. Levy

EXHIBIT A

of

DECLARATION UNDER 35 U.S.C. § 132

**Resume of the Declarant, Paul A. Porter**



**Paul A. Porter, Ph.D.**  
1025 190<sup>th</sup> St.  
Webster City, IA 50595  
515 543-4852 x222

## **Education:**

Ph.D., Animal Science (Dairy Nutrition emphasis), Cornell University, Ithaca, NY. 1987.  
M.S., Animal Science, Cornell University, Ithaca, NY. 1984.  
B.A., Chemistry, Wittenberg University, Springfield, OH. 1981.

## **Professional Experience:**

### **2000-present**

**Dairy Research Manager**, Land O' Lakes Research Farm, Webster City, IA  
Responsible for design, implementation and summary of research trials in the 240-cow dairy herd; present summarized information to the Feed Division and assist in new product development; manage \$700,000 annual budget.

### **1994-2000**

**Dairy Nutritionist and Field Technical Services Manager**, Land O' Lakes Dairy Feed, Sun Prairie, WI  
Provided technical assistance, troubleshooting, product support and training to local cooperatives, their sales staff and their dairy farm clients. Developed sales tools such as spreadsheet economic evaluations and new product information materials.

### **1992-1994**

**Dairy Sales & Consulting**, Young's Livestock Nutritional Services, Canastota, NY  
Provided management and production record analysis & consulting and designed customized feeding programs for dairy farm clients packaged with sales of mineral products.

### **1989-1992**

**Dairy Program Manager**, Countrymark Coop, Delaware, OH  
Provided technical assistance, troubleshooting, product support and training to local cooperatives, their sales staff and their dairy farm clients. Developed sales tools such as spreadsheet economic evaluations and new product information materials.

### **1987-1989**

**Assistant Professor**, Department of Animal Science, Oregon State University, Corvallis  
Conducted applied dairy nutrition research and taught courses in ration balancing, dairy herd management and advanced dairy herd management; acted as liaison to state dairy industry and presented papers at numerous meetings.

### **Memberships:**

American Dairy Science Association  
American Registry of Professional Animal Scientists (ARPAS)



Applicant	: Cindie M. Luhman	
Serial No.	: 09/239,873	
Filed	: January 29, 1999	Group Art Unit: 1616
For	: METHOD AND COMPOSITION FOR ENHANCING MILK COMPONENT CONCENTRATIONS	Examiner: N. Levy
Docket No.	: LL11.12-0040	

EXHIBIT B

of

DECLARATION UNDER 35 U.S.C. § 132

Grant, Roger and Grant, Claire, Grant & Hackh's Chemical Dictionary,  
page 307 (5<sup>th</sup> edition McGraw-Hill Book Company)



er artificial  
materials.  
enol, and

re metals (as  
pound A  
s. Cf. alloy.  
lecules. Cf.

A compound  
f a molecule.  
ctive  
ie dextro-,  
ithin a  
n organic  
id radical  
dded to a  
he results are  
e are

.. atomic  
by the I.  
ization for  
ountries,  
ata,  
s. See  
emperature.  
Applied  
arizes  
ata, atomic  
Applied  
emists (IUB)  
the potency  
(under

ethods, of a  
established.

rrrent.  
ire or tissue.  
molecule.  
sity of a  
t solution of  
of the solute.  
a drug, or  
ds.

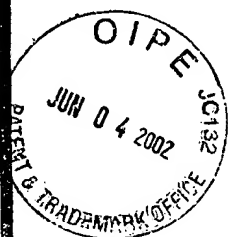
The  
intranuclear

matter Matter  
d, e.g.,

the same  
on occurring  
n Ring  
organic  
densation  
binary)  
rnal oxidation  
C<sub>6</sub>H<sub>4</sub>COOH·

**intramuscular** Inside muscular tissue; as, of an injection.  
**intranuclear** (1) Within an atomic nucleus. (2) Within a molecular ring system. **i. tautomerism** The shifting of a double bond within one or more rings.  
**Intraval sodium** Trademark for thiopental sodium.  
**intravenous** Within veins, e.g., an injection.  
**intravital** (1) Within the living organism. (2) Within a lifetime.  
**introduction** Causing the entry of a different type of atom into an organic molecule, e.g., chlorination.  
**introfaction** A change in the fluidity and specific wetting properties of an impregnating material, due to an introfier.  
**introfier** Impregnation accelerator. A substance that speeds up the penetrating power of fluids.  
**intrusion** Forcing a material into the cavities or pores of a substance.  
**intumescence** (1) Swelling up, especially of certain crystals on heating. (2) Popping, puffing. The violent escape of moisture on heating.  
**inula camphor** Helenin.  
**inulin** (C<sub>6</sub>H<sub>10</sub>O<sub>5</sub>)<sub>n</sub>·2H<sub>2</sub>O. A carbohydrate associated with inulin. Colorless needles, soluble in water.  
**inulic acid** Alantic acid.  
**inulin** C<sub>6</sub>H<sub>11</sub>O<sub>5</sub>(C<sub>6</sub>H<sub>10</sub>O<sub>5</sub>)<sub>n</sub>OH. Alantin, alant starch, dahlin, sinistrin. A polysaccharide from the rhizome of *Inula helenium* or *Dahlia variabilis*. White powder, m. 160 (decomp.), soluble in hot water. Used to measure glomerular filtration rate of kidneys.  
**inulinase\*** An enzyme that endohydrolyzes 2,1-β-D-fructosidic linkages in inulin.  
**in vacuo** In a vacuum, q.v.  
**Invar** Trademark for the ferronickel: Ni 36, steel 64% (carbon content 0.2%), d. 8.0, m. 1500. It has a low coefficient of heat expansion; used for precision instruments.  
**inversion** (1) The turning of a levo to a dextro compound, or vice versa. (2) The change of an isomeric compound to its opposite, as a *cis* to a *trans* compound. (3) The hydrolysis of an optically active disaccharide to 2 optically active monosaccharides; e.g., the hydrolysis of cane sugar to glucose and fructose by dilute acids, alkalies, or enzymes, resulting in a change in the direction and degree of rotation of polarized light. Cf. *Walden inversion*, *Clerget inversion*. (4) In an emulsion of 2 immiscible liquids, the interchange of the internal and external phases. **dipole ~** Symmetrization. The reversal of the normal activity of functional groups in organic chemistry.  
**i. point** The temperature at which i. takes place.  
**invertase** β-D-Fructofuranosidase\*, saccharase, invertin. An enzyme of the pancreatic juice and of yeast, which hydrolyzes terminal, nonreducing β-D-fructofuranoside residues in β-D-fructofuranosides; converts cane sugar into invert sugar.  
**invertin** Invertase.  
**invert soap** A cationic, surface-active detergent, so called because it ionizes oppositely to soap; e.g., quaternary ammonium or sulfonium compounds.  
**invert sugar** Approximately 50% glucose and 50% fructose, obtained by the acid hydrolysis of cane sugar. It is slightly levorotatory, fermentable; it reduces Fehling's solution and is used in brewing. **i. s. solution** A partially inverted solution of sucrose containing at least 62% solids, 3–50% i. s., and equal weights of fructose and glucose.  
**in vitro** Describing a biological reaction which can be performed outside the living organism in the laboratory; as, in a test tube or petri dish, on a microscope slide, etc. Cf. *in vivo*.  
**i. v. fertilization** I.V.F. Fertilization in the laboratory of a

(human) ovum, removed from an ovary, by sperm. (Used in conception of "test tube babies.") See *embryo replacement*.  
**in vivo** Describing a reaction which takes place within the living organism. Cf. *in vitro*.  
**inyoite** 2CaO·3B<sub>2</sub>O<sub>3</sub>·13H<sub>2</sub>O. A native borate (S. California).  
**iod-** See *iodo-*.  
**iodal** Cl<sub>3</sub>·CHO = 421.7. A liquid resembling chloral.  
**iodalbin** A red compound of blood albumin and iodine, of molasseslike odor.  
**iodaniline** Iodoaniline\*.  
**iodate\*** A salt of iodic acid, containing the radical IO<sub>3</sub>.  
**ioeosin** C<sub>20</sub>H<sub>8</sub>O<sub>5</sub>I<sub>4</sub> = 835.9. Erythrosin, tetraiodofluorescein. A red indicator powder, soluble in alcohol (alkalies—rose-red, acids—yellow). **i. solution** A 0.0002% solution of ioeosin in ether. This is added to dilute alkali and titrated until the rose tint passes from the ether into the aqueous solution.  
**iodi-** See *iodo-*.  
**iodic i. acid\*** HIO<sub>3</sub> = 175.9. Metaiodic acid. Colorless rhombs, m. 110, soluble in water. Used as an oxidizing agent; as a reagent for alkaloids, biliary pigments, naphthol, thiocyanates, and guaiacol; in organic synthesis, and for volumetric solutions *per ~* See *periodic acid*. **i. anhydride** Iodine pentaoxide\*.  
**iodide\*** MI<sub>n</sub>. A binary compound of iodine with a metal. **i. ion\*** The I<sup>-</sup> ion.  
**iodimetry** Iodometry.  
**iodinated (<sup>131</sup>I) serum** A sterile solution of human serum albumin, treated with <sup>131</sup>I and freed from iodide; used to diagnose lung conditions; as, small tumors or emboli, and to estimate blood volume.  
**iodine\*** I = 126.9045. Id\* (if I\* is inconvenient). Iodum. A nonmetallic element, at. no. 53, of the halogen group. Rhombic, bluish-black, lustrous plates or scales, d. 4.948, m. 114, b. 184, slightly soluble in water, soluble in alcohol or iodide solutions. Discovered by Courtois (1811) and named after its purple vapors (Greek: *iodēs*, the "violet" and *ion*, "similar"). Obtained from the mother liquor of Chile saltpeter and seaweed ash, and widespread in nature. Valency: usually 1 (iodides\*), or 3 (iodonium\*), or 5 (iodates\*). Used as a reagent in volumetric analysis; in organic synthesis; in the manufacture of iodides, iodates, and iodine preparations; and as an antiseptic and caustic. Used medically (<sup>125</sup>I and <sup>131</sup>I) as sodium iodide and iodinated albumin. I. is also an essential trace element, present in thyroid hormones; deficiency in diet leads to goiter and hypothyroidism. Recommended daily intake 150 μg. **eka ~** Early name for astatine.  
**povidone-~** (C<sub>6</sub>H<sub>9</sub>ON)<sub>n</sub>I. 1-Vinyl-2-pyrrolidinone polymer with iodine. Betadine. An antiseptic (USP, EP, BP). **solution of ~** (1) Lugol solution. (2) Colorless Lugol solution; decolorized with sodium thiosulfate. (3) *Iodine water*, q.v.  
**tincture of ~** An alcoholic 7% iodine solution in 5% potassium iodide solution; an antiseptic (USP).  
**i. acetate\*** IC<sub>2</sub>H<sub>3</sub>O<sub>2</sub> = 185.9. A solid prepared from chlörine dioxide and i. in glacial acetic acid. **i. bromides** IBr, i. monobromide; IBr<sub>3</sub>, i. tribromide; IBr<sub>5</sub>, i. pentabromide. **i. chlorides** ICl, i. monochloride; ICl<sub>3</sub>, i. trichloride. **i. cyanide\*** ICN = 152.9. Cyanogen i. Colorless crystals, m. 146, soluble in water. **i. cycle** See Fig. 15. **i. dioxide\*** IO<sub>2</sub> = 158.9 or I<sub>2</sub>O<sub>4</sub> = 317.8. Yellow powder, decomp. into its elements at 130. **i. disulfide** Sulfur iodide. **i. green** A phenolphthalein dye pH indicator, changing at 1.0 from yellow (acid) to blue-green (alkaline); also stains liquefied xylem in plant tissues. **i. fluoride** See *iodine pentafluoride*. **i. monobromide\*** IBr = 206.8. Purple crystals, m. 36, soluble in water (decomp.). Used



# GRANT & HACKH'S CHEMICAL DICTIONARY

[American, International, European and British Usage]

*Containing the Words Generally Used in Chemistry,  
and Many of the Terms Used in the Related  
Sciences of Physics, Medicine, Engineering,  
Biology, Pharmacy, Astrophysics,  
Agriculture, Mineralogy, etc.*

*Based on Recent Scientific Literature*

FIFTH EDITION

*Completely Revised and Edited by*

**ROGER GRANT**

*M.A., D. de l'U., Ph.D., C. Chem., M.R.S.C. Consultant*

**CLAIRE GRANT**

*M.B., B.S., M.R.C.P.E. Medical Practitioner*

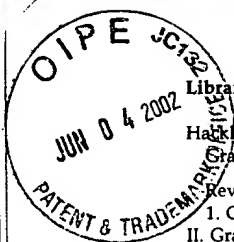
**KINNEY  
& LANGE**

**A PROFESSIONAL ASSOCIATION**

Suite 1500, 625 Fourth Avenue South  
Minneapolis, Minnesota 55415-1659  
(612) 339-1863

**McGRAW-HILL BOOK COMPANY**

New York St. Louis San Francisco Auckland Bogotá  
Hamburg London Madrid Mexico  
Milan Montreal New Delhi Panama  
Paris São Paulo Singapore  
Sydney Tokyo Toronto



Library of Congress Cataloging-in-Publication Data

Hackh, Ingo W. D. (Ingo Waldemar Dagobert), 1890-1938.  
Grant & Hackh's chemical dictionary.

Rev. ed. of: Chemical dictionary. 4th ed. 1969.  
I. Chemistry—Dictionaries. I. Grant, Roger L.  
II. Grant, Claire. III. Title. IV. Title: Grant &  
Hackh's chemical dictionary. V. Title: Chemical  
dictionary.

QD5.H3 1987 540'.3 86-7496  
ISBN 0-07-024067-1

Copyright © 1987 by McGraw-Hill, Inc. All rights reserved.  
Printed in the United States of America. Except as permitted  
under the United States Copyright Act of 1976, no part of this  
publication may be reproduced or distributed in any form or by  
any means, or stored in a data base or retrieval system, without  
the prior written permission of the publisher.

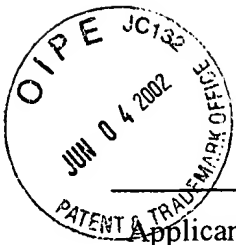
234567890 DOCDOC 943210

ISBN 0-07-024067-1

The previous edition of this book was *Hackh's Chemical Dictionary*,  
4th ed., published by McGraw-Hill in 1969. It was prepared by Dr.  
Julius Grant from a *Chemical Dictionary* compiled by Ingo W. D.  
Hackh. The current, or 5th, edition of this book was prepared by Dr.  
Roger L. Grant, whose father prepared the 4th edition.

*The editors for this book were Betty J. Sun and Susan Thomas,  
the designer was Naomi Auerbach, and the production  
supervisor was Teresa F. Leaden. It was set in Palatino  
by University Graphics, Inc.*

*Printed and bound by R. R. Donnelley & Sons Company.*



Applicant	: Cindie M. Luhman	
Serial No.	: 09/239,873	
Filed	: January 29, 1999	Group Art Unit: 1616
For	: METHOD AND COMPOSITION FOR ENHANCING MILK COMPONENT CONCENTRATIONS	Examiner: N. Levy
Docket No.	: LL11.12-0040	

EXHIBIT C

of

DECLARATION UNDER 35 U.S.C. § 132

Hobson, P.N., The Rumen Microbial Ecosystem,  
pages 461-463 (1988, 1<sup>st</sup> Edition, Elsevier Science Publishers Ltd.)



## Models, Mathematical and Biological, of the Rumen Function

P. N. Hobson\*

*Honorary Research Fellow, Biochemistry Department,  
Marischal College, Aberdeen University, Aberdeen, UK*

*Formerly Head, Microbial Chemistry Department,  
Rowett Research Institute, Aberdeen, UK*

&

J.-P. Jouany

*Laboratoire de la Digestion des Ruminants, INRA Digestion,  
Theix, France*

### TYPES OF MODELS

#### The biological model

In the preceding chapters the functions of the rumen microorganisms as a whole and in the animal, of the microorganisms transferred to a laboratory vessel, and of microorganisms isolated from the rumen mixture acting alone or with one or two others in laboratory apparatus, have been described. It is initially assumed in the experiments *in vitro* that the microbes will react as they do in the rumen. Some comparison of the results with measurements made on the organisms *in vivo* is then made to see how far the initial assumption can be verified. The fact that the reactions *in vitro* do not reproduce those *in vivo* does not necessarily mean that the test organism has no place in the rumen system; it may be that its growth conditions in the rumen have not been properly reproduced in the laboratory, or it may mean that its growth in the rumen is overshadowed by some unisolated organism. On the other hand, the fact that the rumen reactions are apparently reproduced *in vitro* does not always mean that the test organism has a place in the rumen flora and that it is there behaving as in the laboratory experiment. Pure cultures of many organisms may react similarly, as may mixed cultures containing a variety of components.

These cultures may be referred to as 'biological models' of the rumen system. However, they are, probably invariably, reproducing only a part of the rumen reactions. This may be by design; a pure culture or mixed culture of two or three components cannot be expected to reproduce more than the reactions it was

\* Present address: 4 North Deeside Road, Aberdeen AB1 7PL, UK.

designed to study. On the other hand, it may not be safe to assume that a complex mixed culture, such as an artificial rumen, is reproducing all features of the natural rumen because it is reproducing the main features.

One of the assumptions inherent in the tests with pure, or simple defined, cultures is that, if the reactions *in vitro* and *in vivo* are very similar, the test organisms must be those which are carrying out the rumen reactions, or else that any other organisms concerned are very similar to the test organisms, and that the rumen reaction requires these organisms alone. As a corollary, it is assumed that if the complex mixed culture, the artificial rumen, functions in its measured parameters as does the rumen although it is known that some organisms present in the natural rumen are not found in the artificial habitat, then these organisms are not necessary to the proper function of the natural system.

Various experiments, of the type just discussed and others, suggested that the functional rumen population was composed of a limited number of organisms and that the others in the complex population that exists in the actual rumen were unnecessary or there only by chance, possibly kept growing by continued inoculation from the animal's surroundings. Undoubtedly there are some organisms, common to soils and waters for instance, that are always present in the rumen in low numbers but to which, because of their properties and the numbers in which they occur, no reasonable function can be assigned (see Chapter 2). Indeed, their properties may make it reasonable to assume that their apparent growth is only the result of continued inoculation. This assumption is probably quite true for organisms present in only tens or hundreds per ml of rumen contents. It is more difficult to prove this hypothesis for organisms which are present in numbers of some millions per ml, even though this number is only a fraction of 1% of the rumen bacterial population of some  $10^{10}$  per ml.

Whether or not the biological model is reproducing *in vitro* the reactions of the rumen flora, it cannot physically reproduce the rumen and its functions in association with the animal. Because of contamination from its surroundings, the normal animal cannot be used to study the hypothesis of the limited functional rumen population. Thus the idea of using ruminants brought up in a sterile environment, with no rumen microbes, to which could be given a defined rumen flora, came about. This is the 'gnotobiotic lamb' concept. The functions of a rumen population *in toto* could be investigated not only by measuring certain parameters but by assessing others in the ability of the flora to keep the animal alive and healthy. The gnotobiotic lamb is still a 'model' in that it seeks to reproduce the rumen function without reproducing the natural rumen flora. It can also be used to model such things as the natural invasion of the rumen by animal-pathogenic organisms, or the involvement of certain bacteria in rumen malfunction or detoxification of certain toxic feeds (Chapter 14). However, unlike the *in vitro* model, the *in vivo* model must reproduce the whole digestive function to keep the animal alive, even if the main objective is to study only part of the system.

To test the functions of the rumen ciliate protozoa, a rather less rigorously controlled biological model than the gnotobiotic lamb can be used, and the

production of, and described later in the

### The mathematical r

The rumen system is the animal feed, and degraded to various the rumen or pass o of the gut. The mic Liquid, as saliva or thus concerned wit and with rates of de All these changes expressions describ effects of changes quantitative funct mathematical mod then should sugges changes of the desc be impossible or n remembered that t

Over many year try to describe the These expressions bacterial functions of theoretical limi have been put. A si itself with the m fermentor control, is to produce cont for mathematical of the model in de determine the exte cell and in popula model hopes to experiments with t parameters of the what would happ in quantity or qua use to which the n system. The math rumen bacteria in process, and its s

production of, and the results obtained with, the two types of model will be described later in this chapter.

### The mathematical model

The rumen system is an 'open' microbial culture. A mass of microbial nutrients, in the animal feed, enters the rumen at intervals. In the rumen the nutrients are degraded to various extents to different end-products which may be absorbed from the rumen or pass on with undegraded feed components to the more distal portions of the gut. The microorganisms grow, die, lyse or pass on with the undegraded feed. Liquid, as saliva or drinking water, also enters and leaves the rumen. The system is thus concerned with changes in weights, of microbes and feedstuffs and products, and with rates of degradation and synthesis, and passage into and out of the system. All these changes are related, and it should theoretically be possible to make expressions describing the relationships and use these expressions to determine the effects of changes in one factor on another. This is a 'mathematical' model of quantitative functions of the rumen microbes. The first step in making a mathematical model is to be able to describe the system in words or a diagram. This then should suggest the data which will be needed to evaluate quantitatively the changes of the description. The model may not describe the whole system; this may be impossible or not necessary, but if the whole is not described then it should be remembered that the rest of the system may affect the part described.

Over many years various mathematical expressions have been developed which try to describe the growth and metabolism of bacteria under different conditions. These expressions vary in complexity and the extent to which they describe the bacterial functions (see, for instance, Bazin, 1983; Roels, 1983). This is partly because of theoretical limitations and partly because of the uses to which the expressions have been put. A simple description of oxygen uptake rates which does not concern itself with the mechanisms of oxygen metabolism may be quite sufficient in fermentor control, for instance, and one of the reasons for mathematical modelling is to produce control systems for microbial fermentations. Another of the reasons for mathematical modelling of bacterial growth and metabolism is that the success of the model in describing the actual behaviour of bacteria in culture can help to determine the extent of our knowledge of the processes which go on in the bacterial cell and in populations of bacteria. A further, and more usual, reason is that the model hopes to describe the system with such accuracy that it can replace experiments with the actual system and predict what will happen if some controlling parameters of the system are changed. Thus a rumen model might hope to predict what would happen to production of VFA or other metabolites if feed was changed in quantity or quality. However, the complexity of a rumen model will depend on the use to which the model is to be put as well as the state of knowledge of the microbial system. The mathematical model is like the *in vitro* fermentations with pure or mixed rumen bacteria in that it can describe the whole or only a part of the digestive process, and its success is not dependent on describing accurately the whole.

# THE RUMEN MICROBIAL ECOSYSTEM

*Edited by*

**P. N. HOBSON**

*Honorary Research Fellow, Biochemistry Department,  
Marischal College, Aberdeen University,  
Aberdeen, UK*

*Formerly Head, Microbial Chemistry Department,  
Rowett Research Institute, Aberdeen, UK*



**ELSEVIER APPLIED SCIENCE**  
LONDON and NEW YORK

QK  
171  
.R85



ELSEVIER SCIENCE PUBLISHERS LTD  
Crown House, Linton Road, Barking, Essex IG11 8JU, England

*Sole Distributor in the USA and Canada*  
ELSEVIER SCIENCE PUBLISHING CO., INC.  
52 Vanderbilt Avenue, New York, NY 10017, USA

WITH 68 TABLES AND 48 ILLUSTRATIONS

© 1988 ELSEVIER SCIENCE PUBLISHERS  
(except Chapter 10: © Government of Canada)

British Library Cataloguing in Publication Data

The Rumen microbial ecosystem.  
1. Ruminants. Rumen. Microflora  
I. Hobson, P.N.  
599.73'504132

Library of Congress Cataloging in Publication Data

The Rumen microbial ecosystem.  
1. Rumen—Microbiology. I. Hobson, P. N.  
QR171.R85R86 1988 599.73'504132 88-16424

ISBN 1-85166-188-3

No responsibility is assumed by the Publisher for any injury and/or damage to persons or property as a matter of products liability, negligence or otherwise, or from any use or operation of any methods, products, instructions or ideas contained in the material herein.

#### Special regulations for readers in the USA

This publication has been registered with the Copyright Clearance Center Inc. (CCC), Salem, Massachusetts. Information can be obtained from the CCC about conditions under which photocopies of parts of this publication may be made in the USA. All other copyright questions, including photocopying outside the USA, should be referred to the publisher.

All rights reserved. No part of this publication may be reproduced, stored in a retrieval system, or transmitted in any form or by any means, electronic, mechanical, photocopying, recording, or otherwise, without the prior written permission of the publisher.

Printed in Great Britain by Galliard (Printers) Ltd, Great Yarmouth

QR  
171  
R85  
R86  
1988

Ruminants have and have provided kinds of climates vegetation from

The ability to rumen, and this was known 100 c not until 40 or 5 digestion of veg

In the late 194 nutrition of the said to have a d

Progress was conversion of v bacteria and pro or investigated in in the rumen an reactions were kn rumen microbiol

*The Rumen and*

Since then, pu with other scienti but new techniq microorganisms, non-ruminant I demonstrated.

The rumen is a many reactions. I habitats other the



Applicant	: Cindie M. Luhman	
Serial No.	: 09/239,873	
Filed	: January 29, 1999	Group Art Unit: 1616
For	: METHOD AND COMPOSITION FOR ENHANCING MILK COMPONENT CONCENTRATIONS	Examiner: N. Levy
Docket No.	: LL11.12-0040	

EXHIBIT D

of

DECLARATION UNDER 35 U.S.C. § 132

Hobson, P.N. and Stewart C.S., The Rumen Microbial Ecosystem,  
pages 661-662 (1997, 2<sup>nd</sup> Edition, Chapman and Hall)



## 15 Biological models of the rumen function

P. N. HOBSON

Department of Molecular and Cell Biology, University of  
Aberdeen, Aberdeen, UK

and

G. FONTY

Laboratoire de Microbiologie, Institut National de la  
Recherche Agronomique, CR Clermont-Ferrand-Theix,  
Saint-Genès-Champanelle, France

In the preceding chapters the functions of the rumen microorganisms as a whole, in the animal or transferred to a laboratory vessel, and of microorganisms isolated from the rumen mixture acting alone or with one or two others in laboratory apparatus, have been described. In these experiments, it is assumed that the microorganisms are reproducing, *in toto* or in some specific metabolic pathway, the reactions that occur in the rumen. The results of such experiments suggested that this was indeed so and that the rumen reactions could be carried out by mixtures of a comparatively small number of species of anaerobic bacteria, the other bacteria being 'contaminants' introduced with the feed and air and being, at least in some cases, kept in small populations by continued inoculating from the surroundings of the animals. As mentioned later, the ciliate protozoa were not considered to be essential for the rumen function and the anaerobic fungi had not been recognized at the start of the experiments to be described here.

However, although experiments *in vitro* might reproduce metabolic pathways, they could not always reproduce them quantitatively and they could not demonstrate that these reactions were sufficient to keep a ruminant alive and growing; also, they could not completely reproduce the conditions in the rumen. Mechanical means cannot reproduce the rumen mixing and a dialysis membrane cannot reproduce the metabolizing rumen wall in the absorption of molecules, rumination as an aid to comminution of fibres, etc. The only 'container' that reproduces the rumen is the rumen. Thus, the idea of using the ruminant itself as a 'container' for experiments on the functions

of defined rumen floras came about. Such a ruminant can be described as a 'biological model' by analogy with the 'mathematical model' where the rumen flora is described in simplified verbal and mathematical terms.

However, in the normal animals, although the rumen is sterile at birth it is rapidly inoculated and ends up with the 'normal' rumen flora and fauna. The inoculum organisms come from the air, feedstuffs and bodily contact with nearby animals (Chapters 2, 3 and 5). While inoculation by ciliate protozoa can be prevented by keeping young animals some distance from older animals (Chapter 5), as the relatively large protozoa can travel only a short distance in air currents, rumen bacteria can be carried for long distances and can be found in samples of air from animal houses (Mann, 1963). Spatial isolation, therefore, cannot prevent the development of a rumen bacterial population in young animals. Therefore, the only way in which the rumen can be used as a model system is to isolate it, and thus the animal, in a sterile atmosphere from birth. This will keep the rumen uninoculated and so in a position to be inoculated with a population of known bacteria: to become a 'gnotobiotic' ruminant. Experiments described elsewhere (Chapters 5 and 13) have shown that the ciliate protozoa are not necessary in a rumen population that will ensure adequate growth of a young animal. On the other hand, bacteria are essential for the survival of the protozoa (Chapter 3). Since it is difficult or impossible to obtain cultures of protozoa with no, or only defined bacterial populations, experiments with gnotobiotic ruminants have concentrated on providing a defined rumen bacterial population without protozoa. Prevention of protozoal growth is also a way of manipulating the rumen microbial population, and the subject has thus been mentioned in Chapter 13.

### 15.1 Defined bacterial populations without protozoa

Isolation in a sterile environment is, of course, what has been done in many experiments with 'gnotobiotic' or 'germ-free' animals. There is, however, a big difference between the mice, rats, monkeys and other animals that are used in the usual gnotobiotic experiments, and ruminants. The former animals digest food by means of enzymes in gut secretions, and microbial digestion is a minor part of the digestion and not strictly necessary for the life of the animal. In the adult ruminant the main digestive process is microbial and the ruminant cannot live without a functioning rumen even if fed on a diet which could support a non-ruminant germ-free animal. Normally, the ruminant at birth has a sterile gut like any other animal, but bacteria begin to colonize the internal and external surfaces of the animal within a few minutes of birth and a gut flora soon develops (Fonty *et al.*, 1987a; Anderson *et al.*, 1987). This flora is initially similar to that of any other milk-fed animal, and it is not strictly necessary for digestion; so, as

with other mammals, a rumen must develop under sterile conditions; function and size of the rumen and the adult rumen flora and fauna of a young ruminant can be determined. Therefore, the experiments with gnotobiotic ruminants to determine the factors that control the rumen flora must produce the fermentation products themselves, and the final products are volatile fatty acids (VFAs) and vitamins at rates sufficient for the animal. A bigger problem than estimating the rate of growth on products of rumen enzymes. The fact that the rumen must keep the animal, and so the experiments *in vitro* preclude tests only on the bacterial processes by inoculating the rumen with a defined carbon, nitrogen and other undefined polymers and chemicals of the *in vitro* type, and other bacteria must be added to the bacterial 'culture'. The gnotobiotic rumen is growing only poorly, show with the rumen; by growing, it is possible to determine necessary for the rumen to function. A functioning rumen flora and chemical and bacteriological pathways in the rumen. Experiments on the factors in control of 'invading' pathogens.

In this section 'gnotobiotic' ruminants with no known gut flora are compared with a known gut flora and a rumen. In the first case, the animal is a ruminant recently been used to determine the rumen flora of an animal inoculated with a defined rumen flora. An animal inoculated with a defined rumen flora as 'meroxenic'. In this case, the rumen can also be used to describe the rumen flora of a mother in a conventional rumen a few hours or a few days.



# The Rumen Microbial Ecosystem

*Edited by*

**P.N. HOBSON**

*Department of Molecular and Cell Biology  
University of Aberdeen*

*and*

**C.S. STEWART**

*The Nutrition Division  
The Rowett Research Institute  
Aberdeen*

**GOPHER, FTP OR EMAIL:**

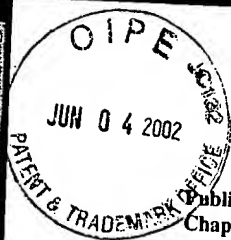
A service of **ITP**<sup>®</sup>



**BLACKIE ACADEMIC & PROFESSIONAL**

An Imprint of Chapman & Hall

London · Weinheim · New York · Tokyo · Melbourne · Madras



Published by Blackie Academic & Professional, an imprint of  
Chapman & Hall, 2-6 Boundary Row, London SE1 8HN

Chapman & Hall, 2-6 Boundary Row, London SE1 8HN, UK

Chapman & Hall GmbH, Pappelallee 3, 69469 Weinheim, Germany

Chapman & Hall USA, 115 Fifth Avenue, New York, NY 10003, USA

Chapman & Hall Japan, ITP-Japan, Kyowa Building, 3F, 2-2-1  
Hirakawacho, Chiyoda-ku, Tokyo 102, Japan

DA Book (Aust.) Pty Ltd, 648 Whitehorse Road, Mitcham 3132, Victoria,  
Australia

Chapman & Hall India, R. Seshadri, 32 Second Main Road, CIT East,  
Madras 600 035, India

First edition 1988

Second edition 1997

© 1997 Chapman & Hall

Typeset in 10/12pt Times New Roman by Best-set Typesetter Ltd., Hong Kong  
Printed in Great Britain by St Edmundsbury Press, Bury St Edmunds, Suffolk

ISBN 0 7514 0366 0

Apart from any fair dealing for the purposes of research or private study,  
or criticism or review, as permitted under the UK Copyright Designs and  
Patents Act, 1988, this publication may not be reproduced, stored, or  
transmitted, in any form or by any means, without the prior permission in  
writing of the publishers, or in the case of reprographic reproduction only  
in accordance with the terms of the licences issued by the Copyright  
Licensing Agency in the UK, or in accordance with the terms of licences  
issued by the appropriate Reproduction Rights Organization outside the  
UK. Enquiries concerning reproduction outside the terms stated here  
should be sent to the publishers at the London address printed on this  
page.

The publisher makes no representation, express or implied, with regard  
to the accuracy of the information contained in this book and cannot  
accept any legal responsibility or liability for any errors or omissions that  
may be made.

A catalogue record for this book is available from the British Library

Library of Congress Catalog Card Number: 96-80242

Printed on acid-free text paper, manufactured in accordance with ANSI/  
NISO Z39.48-1992 (Permanence of Paper).

QR171  
R85  
R86  
1999

Dedicated to Bot



Applicant : Cindie M. Luhman

Serial No. : 09/239,873

Filed : January 29, 1999

For : METHOD AND COMPOSITION FOR  
ENHANCING MILK COMPONENT  
CONCENTRATIONS

Docket No. : LL11.12-0040

Group Art Unit: 1616

Examiner: N. Levy

EXHIBIT E

of

DECLARATION UNDER 35 U.S.C. § 132

Hobson, P.N. and Stewart, C.S., The Rumen Microbial Ecosystem,  
page 496 (1997, 2<sup>nd</sup> Edition, Chapman and Hall)

JUN 04 2002

PATENT &amp; TRADEMARK OFFICE

JUN 04 2002

PATENT &amp; TRADEMARK OFFICE

496

## COMPARTMENTATION IN THE RUMEN

microorganisms are found in suspension, in association with the solid digesta, and adherent to the rumen wall (Cheng *et al.*, 1995; Figures 12.2 and 12.3), there is clearly more than one microbial compartment within this organ.

The rumen has a complex structure and the contents are heterogeneous, consisting primarily of a microbial suspension in free liquid, a solid mass of digesta, and a gas phase. Each of these entities is complex; the properties of microorganisms in suspension deep in the rumen might be different from those of microorganisms close to the rumen wall (Cheng and Costerton, 1980). The solid mass of digesta is certainly heterogeneous, and even the gas phase is complex, consisting of a large gas space (gas-cap), smaller pockets of gas entrapped within the solid mass, and the dissolved gas. Many properties of individual parts of the microbial system of the rumen have been investigated, but often the investigations were governed by the ease of experimentation rather than the importance of the given component. This is why the complex, and relatively inaccessible, semi-solid mass of digesta has received so little attention. In addition, worthwhile investigations were hampered by a lack of a simple conceptual basis for dealing with such a complex system.

The microbial populations of the rumen are effectively subdivided in Cheng's 'three population' theory (Cheng and Costerton, 1980) into those organisms free in the rumen fluid, those associated with feed particles and those associated with the rumen wall (Figures 12.2 and 12.3). Czerkawski (1980) subdivided the second population into those organisms firmly associated with particulate food (degradative compartment) and those more reversibly associated (shuttle compartment). In the functioning rumen, the compartments are clearly in a dynamic equilibrium, as ruminal microbes adhering to and detaching from their substrata (feed particles, or rumen epithelial cells) are effectively leaving and re-entering the fluid compartment.

Compartmentation within the rumen is not present immediately at birth, but evolves in the newborn ruminant over a period of approximately 20 days (Figure 12.4). Compartment 4 is the first to establish. Bacterial colonization of gut tissue in neonatal calves is initiated within 24 h of birth. Compartments 2 and 3 become established soon thereafter, often within 4 days of birth (Fonty *et al.*, 1987). Diversity of microbial members of compartments 1, 2 and 3 increases tremendously as fungal and protozoal populations become established in the rumen, at 8–10 days and 12–20 days of age, respectively. Once established, the microbial populations of each compartment are in general inherently stable and can be altered only by profound changes in nutrient substrata (e.g. transition in the ruminant's diet from forage to concentrate) or by antimicrobial agents (e.g. ionophores) which target specific populations.

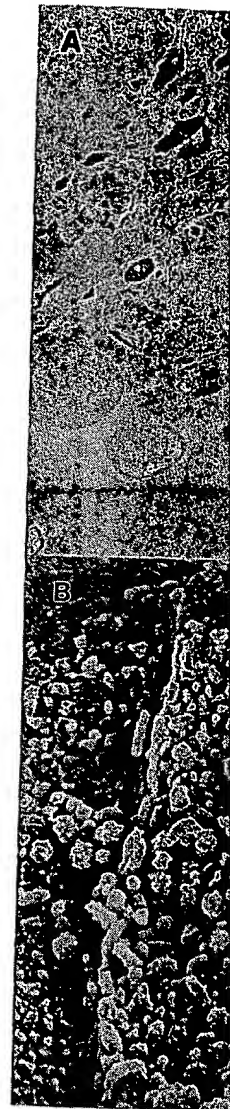


Figure 12.3 The rumen wall in summer from scanning (A) and scanning (B) electron microscopy. A carbohydrate matrix is visible in the summer.





# The Rumen Microbial Ecosystem

*Edited by*

**P.N. HOBSON**

*Department of Molecular and Cell Biology  
University of Aberdeen*

*and*

**C.S. STEWART**

*The Nutrition Division  
The Rowett Research Institute  
Aberdeen*

**Gopher, FTP or Email:**

A service of **ITP**<sup>®</sup>



**BLACKIE ACADEMIC & PROFESSIONAL**

An Imprint of Chapman & Hall

London · Weinheim · New York · Tokyo · Melbourne · Madras

Published by Blackie Academic & Professional, an imprint of  
Chapman & Hall, 2-6 Boundary Row, London SE1 8HN

Chapman & Hall, 2-6 Boundary Row, London SE1 8HN, UK

Chapman & Hall GmbH, Pappelallee 3, 69469 Weinheim, Germany

Chapman & Hall USA, 115 Fifth Avenue, New York, NY 10003, USA

Chapman & Hall Japan, ITP-Japan, Kyowa Building, 3F, 2-2-1  
Hirakawacho, Chiyoda-ku, Tokyo 102, Japan

DA Book (Aust.) Pty Ltd, 648 Whitehorse Road, Mitcham 3132, Victoria,  
Australia

Chapman & Hall India, R. Seshadri, 32 Second Main Road, CIT East,  
Madras 600 035, India

First edition 1988

Second edition 1997

© 1997 Chapman & Hall

Typeset in 10/12pt Times New Roman by Best-set Typesetter Ltd., Hong Kong  
Printed in Great Britain by St Edmundsbury Press, Bury St Edmunds, Suffolk

ISBN 0 7514 0366 0

Apart from any fair dealing for the purposes of research or private study,  
or criticism or review, as permitted under the UK Copyright Designs and  
Patents Act, 1988, this publication may not be reproduced, stored, or  
transmitted, in any form or by any means, without the prior permission in  
writing of the publishers, or in the case of reprographic reproduction only  
in accordance with the terms of the licences issued by the Copyright  
Licensing Agency in the UK, or in accordance with the terms of licences  
issued by the appropriate Reproduction Rights Organization outside the  
UK. Enquiries concerning reproduction outside the terms stated here  
should be sent to the publishers at the London address printed on this  
page.

The publisher makes no representation, express or implied, with regard  
to the accuracy of the information contained in this book and cannot  
accept any legal responsibility or liability for any errors or omissions that  
may be made.

A catalogue record for this book is available from the British Library

Library of Congress Catalog Card Number: 96-80242

Printed on acid-free text paper, manufactured in accordance with ANSI/  
NISO Z39.48-1992 (Permanence of Paper).

QR171  
R85  
R86  
1559

Dedicated to B b

Applicant	: Cindie M. Luhman	
Serial No.	: 09/239,873	
Filed	: January 29, 1999	Group Art Unit: 1616
For	: METHOD AND COMPOSITION FOR ENHANCING MILK COMPONENT CONCENTRATIONS	Examiner: N. Levy
Docket No.	: LL11.12-0040	

EXHIBIT F

of

DECLARATION UNDER 35 U.S.C. § 132

Hobson, P.N. and Stewart, C.S., The Rumen Microbial Ecosystem,  
page 518 (1997, 2<sup>nd</sup> Edition, Chapman and Hall)

In multicellular organisms, the cells differentiate in response to the function of a particular tissue. Some cells remain fixed to form definite functional structures (e.g. muscle cells), while others become mobile (e.g. blood cells). The integrity of this complex system is maintained by connective tissues. The microbial system of the rumen is not a simple fermentation vat filled with randomly distributed mixtures of microorganisms. It is a highly structured and functionally compartmented system, on a par with any of the organs of the advanced multicellular organism. Significant progress in the field of rumen metabolism is contingent on appreciation of the rumen and its microbial ecosystem in this manner.

## References

- stir the contents of a round-bottomed flask. On the contrary, the raft of digesta in animals on roughage diets is more or less immobilized and the overall structure appears to favour minimal movement of the solid mass of digesta. This must be advantageous, because it is unlikely to disturb the balance of the animal, in which the rumen contents may account for up to one-fifth of its weight and whose survival in the wild state depends so much on its agility. The immobilization of the raft of fibrous digesta and its use as a continuously renewable double-sided filter (Czerkawski, 1986b) ensures its controlled and predictable throughput in an incredibly complicated organ. The compartmentation of the microbial population in the rumen is an indispensable and integral part of this system.
- In multicellular organisms, the cells differentiate in response to the function of a particular tissue. Some cells remain fixed to form definite functional structures (e.g. muscle cells), while others become mobile (e.g. blood cells). The integrity of this complex system is maintained by connective tissues. The microbial system of the rumen is not a simple fermentation vat filled with randomly distributed mixtures of microorganisms. It is a highly structured and functionally compartmented system, on a par with any of the organs of the advanced multicellular organism. Significant progress in the field of rumen metabolism is contingent on appreciation of the rumen and its microbial ecosystem in this manner.
- ## References
- Abe, M. and Iriki, T. (1989). Usi no diaiichiinai zenmouchuu noudo no tokuina nisshyhendou to soren kakawaru youin ni tuite. *Proc. Jap. Soc. Anim. Nutr. Metabol.*, **33**, 77-96.
- Allen, M. S. and Mertens, D. R. (1988). Evaluating constraints on fiber digestion by rumen microbes. *J. Nutr.*, **118**, 261-70.
- Bae, H. D., McAllister, T. A., Kokko, E. G. *et al.* (1997) Effect of silica on the colonization of rice straw by ruminal bacteria. *Anim. Fd Sci. Technol.* (in press).
- Bauchop, T. (1979). The rumen ciliate *Epidinium* in primary degradation of plant tissues. *Appl. Environ. Microbiol.*, **37**, 1217-23.
- Beauchemin, K. A., McAllister, T. A., Dong, Y. *et al.* (1994). Effects of mastication on digestion of whole cereal grains by cattle. *J. Anim. Sci.*, **72**, 236-46.
- Blanchart, G., Durand, M., Barry, J. L. *et al.* (1989). Interets et limites des fermenteurs a flux semi-continu de type Rusitec dans l'etude des fermentations du rumen. *Ann. Zootech.*, **38**, 285-314.
- Bogaert, C., Gomez, L., Jouany, J. P. and Jeminet, G. (1989). Effects of the ionophore antibiotics lasalocid and cationomycin on ruminal fermentation in vitro (RUSITEC). *Anim. Feed. Sci. Technol.*, **27**, 1-15.
- Bonhomme, A. (1990). Rumen ciliates: their metabolism and relationships with bacteria and their hosts. *Anim. Feed. Sci. Technol.*, **30**, 203-66.
- Briesacher, S. L., May, T., Grigsby, K. N. *et al.* (1991). Use of DNA probes to monitor nutritional effects on ruminal prokaryotes and *Fibrobacter succinogenes* S85. *J. Anim. Sci.*, **70**, 289-95.
- Brink, D. R., Lowry, S. R., Stock, R. A. and Parrott, J. C. (1990). Severity of liver abscesses and efficiency of feed utilization of feedlot cattle. *J. Anim. Sci.*, **68**, 1201-7.
- Brock, F. M., Forsberg, C. W. and Buchanan-Smith, J. G. (1982). Proteolytic activity of rumen microorganisms 561-9.
- Cheng, K.-J. and Costerton of plant material, urea *Ruminants*, ed. Y. Ruck
- Cheng, K.-J. and Wallace, the rumen wall and the 553-7.
- Cheng, K.-J., Fay, J. P., F the digestion of fresh le 25.
- Cheng, K.-J., Stewart, C. S of bacteria involved in 120.
- Cheng, K.-J., Forsberg, C. physiology of feed degr *Metabolism in Ruminant* Toronto, pp. 595-624.
- Cheng, K.-J., McAllister, T microflora of the rumen
- Cheng, K.-J., McAllister, T tract. In *Microbial Biofil* sity Press, Cambridge, p
- Cherney, J. H. (1990). Nor lucose utilization. In *Mic* tion by Ruminants, ed. I Science Publishing Com
- Craig, A. M. (1995). Detc *Ruminant Physiology*: Engelhardt, S. Leonhar Stuttgart, pp. 271-88.
- Craig, W. M., Brown, D. R of fluid and particulate r
- Craig, W. M., Broderick, associated with the parti
- Czerkawski, J. W. (1980). C
- Czerkawski, J. W. (1983). 18.
- Czerkawski, J. W. (1986a). 62.
- Czerkawski, J. W. (1986b). the rumen. In *Nuclear an* tional Atomic Energy A
- Czerkawski, J. W. and Br fermentation *in vitro*. La
- Czerkawski, J. W. and Br rumen simulation techni
- Czerkawski, J. W. and Br simulation technique (Ru 217-28.
- Czerkawski, J. W. and Br simulation technique (Ru 229-45.
- Czerkawski, J. W. and Br 3.5.1.5) activity in the ru
- Davies, D. R., Theodorou, l on the survival of anaero *Let.*, **106**, 59-64.

# The Rumen Microbial Ecosystem

*Edited by*

**P.N. HOBSON**

*Department of Molecular and Cell Biology  
University of Aberdeen*

and

**C.S. STEWART**

*The Nutrition Division  
The Rowett Research Institute  
Aberdeen*

**GOPHER, FTP OR EMAIL:**

A service of **ITP**<sup>®</sup>



**BLACKIE ACADEMIC & PROFESSIONAL**

An Imprint of Chapman & Hall

London · Weinheim · New York · Tokyo · Melbourne · Madras

Published by Blackie Academic & Professional, an imprint of  
Chapman & Hall, 2-6 Boundary Row, London SE1 8HN

Chapman & Hall, 2-6 Boundary Row, London SE1 8HN, UK

Chapman & Hall GmbH, Pappelallee 3, 69469 Weinheim, Germany

Chapman & Hall USA, 115 Fifth Avenue, New York, NY 10003, USA

Chapman & Hall Japan, ITP-Japan, Kyowa Building, 3F, 2-2-1  
Hirakawacho, Chiyoda-ku, Tokyo 102, Japan

DA Book (Aust.) Pty Ltd, 648 Whitehorse Road, Mitcham 3132, Victoria,  
Australia

Chapman & Hall India, R. Seshadri, 32 Second Main Road, CIT East,  
Madras 600 035, India

First edition 1988

Second edition 1997

© 1997 Chapman & Hall

Typeset in 10/12pt Times New Roman by Best-set Typesetter Ltd., Hong Kong  
Printed in Great Britain by St Edmundsbury Press, Bury St Edmunds, Suffolk

ISBN 0 7514 0366 0

Apart from any fair dealing for the purposes of research or private study,  
or criticism or review, as permitted under the UK Copyright Designs and  
Patents Act, 1988, this publication may not be reproduced, stored, or  
transmitted, in any form or by any means, without the prior permission in  
writing of the publishers, or in the case of reprographic reproduction only  
in accordance with the terms of the licences issued by the Copyright  
Licensing Agency in the UK, or in accordance with the terms of licences  
issued by the appropriate Reproduction Rights Organization outside the  
UK. Enquiries concerning reproduction outside the terms stated here  
should be sent to the publishers at the London address printed on this  
page.

The publisher makes no representation, express or implied, with regard  
to the accuracy of the information contained in this book and cannot  
accept any legal responsibility or liability for any errors or omissions that  
may be made.

A catalogue record for this book is available from the British Library

Library of Congress Catalog Card Number: 96-80242

♾ Printed on acid-free text paper, manufactured in accordance with ANSI/  
NISO Z39.48-1992 (Permanence of Paper).

QR171  
R85  
R86  
1999

Dedicated to Bot

Applicant	: Cindie M. Luhman	
Serial No.	: 09/239,873	
Filed	: January 29, 1999	Group Art Unit: 1616
For	: METHOD AND COMPOSITION FOR ENHANCING MILK COMPONENT CONCENTRATIONS	Examiner: N. Levy
Docket No.	: LL11.12-0040	

# EXHIBIT G

of

## DECLARATION UNDER 35 U.S.C. § 132

Hobson, P.N., The Rumen Microbial Ecosystem,  
page 428 (1988, 1<sup>st</sup> Edition, Elsevier Science Publishers Ltd.)

optimization. Compounds resulting in a generally lower microbial fermentation activity in the rumen are not interesting, and a high degree of specificity is desirable. However, the realization that the rumen fermentation is a very complex system of interrelated reactions makes a specific action by a particular additive almost impossible to obtain. A compound may possibly have a very specific direct effect, but the overall effect will be dictated by the changed interrelations within the whole system of rumen and indeed ruminant metabolism. For example, the use of methane inhibitors such as chloral hydrate or BES, specifically toxic only for the unique group of methane bacteria, induces a chain reaction with final effects on the pattern of volatile fatty acid production and even on degradation of certain amino acids.

It is striking that, with practically all additives (rumen manipulators), reducing equivalents (2H) are diverted from methane to propionate, and this is associated with effects on amino acid metabolism. This also explains why, in most manipulated situations, acetic acid increases are rarely seen. The complexity of interrelations within the system of rumen metabolism also includes microbial growth yield and digesta kinetics, which makes the study of rumen optimization a very laborious task. Furthermore, not only the effects on the system of rumen metabolism have to be considered but, because the sites of digestion are often changed, the effects on lower-digestive-tract metabolism should be determined, as well as possible effects at the ruminant tissue level. As adaptation to certain compounds has been observed, so effects *in vivo*, including feedlot performance, should be studied in long-term experiments. When experiments also include toxicological and even ecological aspects, the burden of the experiments for both researcher and experimental animal (multiple cannulae in the digestive tract) becomes considerable. Table 1 shows that there is ample choice of compounds interfering with rumen metabolism; for most compounds, however, experimental data on actions are limited and sometimes contradictory. The complexity of the rumen and the ruminant systems to be manipulated is the main reason for variability and contradiction in experimental results. Much more work to comprehend and rationalize this complexity is needed before safe and efficient application of rumen manipulation can be considered and recommended.

#### ACKNOWLEDGEMENT

Our research group is financially supported by the IWONL, Brussels, Belgium.

#### REFERENCES

- Adams, D. C., Galyean, M. L., Kiesling, H. E., Wallace, J. D. & Finkner, M. D. (1981). Influence of viable yeast culture, sodium bicarbonate and monensin on liquid dilution rate, rumen fermentation and feedlot performance of growing steers and digestibility in lambs. *J. Anim. Sci.*, **53**, 780-9.

- Anderson, P. H. monensin or  
Armentano, L. E. glucose and  
*Nutr.*, **113**, 1.  
Baldwin, K. A., secondary ar  
production b  
Baldwin, K. A. dimethyldode  
acid producti  
Banks, W., Clapp forms of dieta  
387-95.  
Barao, S. M., Berg or in combinat  
*Anim. Sci.*, **61**  
Barclay, R. A., Fa on performanc  
*Nutr. Rep. Int.*  
Bartley, E. E., Her monensin/orlas  
feed efficiency.  
Bauchop, T. & Mo in both the abse  
1103-10.  
Beede, D. K. & Fa fatty acid prod  
Beede, D. K. & Fa ruminal pH, laci  
acidosis. *J. Anim*  
Bergen, W. G. & B mode of action.  
Berger, J., Rachlin, isolation of three  
5295-5303.  
Brent, B. E. & Bartle  
Brethour, J. R. & Ch rations. *J. Anim.*  
Brock, F. M., Forsbe microorganisms  
Broderick, G. A. & B by ruminal micro  
Broudiscou, L., Van d'huile de soja dar  
la cellulose. *Repr*  
Brown, D. L. & Hogu milk composition  
Brown, H., Elliston, Tylosin phosphate  
weight gains and f  
Bryant, M. P. & Robi culturable ruminal



# THE RUMEN MICROBIAL ECOSYSTEM

*Edited by*

**P. N. HOBSON**

*Honorary Research Fellow, Biochemistry Department,  
Marischal College, Aberdeen University,  
Aberdeen, UK*

*Formerly Head, Microbial Chemistry Department,  
Rowett Research Institute, Aberdeen, UK*



**ELSEVIER APPLIED SCIENCE**  
LONDON and NEW YORK

QK  
171  
.R85

ELSEVIER SCIENCE PUBLISHERS LTD  
Crown House, Linton Road, Barking, Essex IG11 8JU, England

*Sole Distributor in the USA and Canada*  
ELSEVIER SCIENCE PUBLISHING CO., INC.  
52 Vanderbilt Avenue, New York, NY 10017, USA

WITH 68 TABLES AND 48 ILLUSTRATIONS

© 1988 ELSEVIER SCIENCE PUBLISHERS  
(except Chapter 10: © Government of Canada)

**British Library Cataloguing in Publication Data**

The Rumen microbial ecosystem.  
1. Ruminants. Rumen. Microflora  
I. Hobson, P.N.  
599.73'504132

**Library of Congress Cataloging in Publication Data**

The Rumen microbial ecosystem.  
1. Rumen—Microbiology. I. Hobson, P. N.  
QR171.R85R86 1988 599.73'504132 88-16424

ISBN 1-85166-188-3

No responsibility is assumed by the Publisher for any injury and/or damage to persons or property as a matter of products liability, negligence or otherwise, or from any use or operation of any methods, products, instructions or ideas contained in the material herein.

**Special regulations for readers in the USA**

This publication has been registered with the Copyright Clearance Center Inc. (CCC), Salem, Massachusetts. Information can be obtained from the CCC about conditions under which photocopies of parts of this publication may be made in the USA. All other copyright questions, including photocopying outside the USA, should be referred to the publisher.

All rights reserved. No part of this publication may be reproduced, stored in a retrieval system, or transmitted in any form or by any means, electronic, mechanical, photocopying, recording, or otherwise, without the prior written permission of the publisher.

Printed in Great Britain by Galliard (Printers) Ltd, Great Yarmouth

QR  
171  
.R85  
R86  
1988

Ruminants have and have provided kinds of climates vegetation from

The ability to rumen, and this was known 100 c not until 40 or 5 digestion of vega

In the late 194 nutrition of the : said to have a d

Progress was conversion of v bacteria and pro or investigated in in the rumen an reactions were kn rumen microbiol *The Rumen and*

Since then, pu with other scienti but new techniqu microorganisms, non-ruminant I demonstrated.

The rumen is a many reactions. E habitats other tha

Applicant	: Cindie M. Luhman	
Serial No.	: 09/239,873	
Filed	: January 29, 1999	Group Art Unit: 1616
For	: METHOD AND COMPOSITION FOR ENHANCING MILK COMPONENT CONCENTRATIONS	Examiner: N. Levy
Docket No.	: LL11.12-0040	

# EXHIBIT H

of

## DECLARATION UNDER 35 U.S.C. § 132

Hobson, P.N. and Stewart C.S., The Rumen Microbial Ecosystem,  
pages 586-587 (1997, 2<sup>nd</sup> Edition, Chapman and Hall)

digestion, microbial protein synthesis and vitamin B<sub>12</sub> synthesis, has been documented by single additions of minerals to fermentations *in vitro* (Durand and Kawashima, 1980). It is difficult to extrapolate the *in vitro* observations to *in vivo* situations, because of the influence of a number of other factors, such as solubility of minerals, adaptability of microbes, and interaction among minerals.

Manipulation of ruminal fermentation with the use of minerals requires caution, because the margin between optimal, stimulatory concentration and inhibitory concentration is narrow (Mackie and Therion, 1984; Durand and Komisarczuk, 1988). Additionally, excessive concentration of one mineral may affect the utilization of other minerals by the microbes and the host.

*Four- and five-carbon VFAs, phenylpropanoic and phenylacetic acids.* Isobutyric acid (IBA), isovaleric acid (IVA), 2-methylbutyric acid (2MBA) (branched-chain) and valerate (straight-chain), popularly called 'isoacids', are required absolutely by or stimulate growth of several rumen bacterial species, particularly the fibrolytic organisms (Allison, 1963; Bryant, 1973). These acids, IBA, IVA, 2-MBA and valerate, are fermentation products of valine, leucine, isoleucine and proline, respectively (Allison, 1963). Valerate could also be produced from carbohydrate fermentation. Cellulolytics and some non-cellulolytics are incapable of taking up the branched-chain amino acids and, therefore, must synthesize them from ammonia and branched-chain VFAs (Dehority *et al.*, 1967). Also, these acids serve as precursors for the biosynthesis of branched, long-chain fatty acids and aldehydes of the membrane lipids of bacteria (Bryant, 1973).

Several *in vitro* fermentation studies have shown the importance of the isoacids in fiber digestion (Andries *et al.*, 1987). Addition of branched-chain amino acids (leucine and isoleucine) has also been shown to stimulate fiber

**Table 13.20** Effects of carbon-4 and carbon-5 fatty acids (straight or branched) on *in vitro* wheat straw cell wall digestion and ammonia concentration

Addition	Cell wall digestion (%)	Ammonia concentration (mg l <sup>-1</sup> )
None	17.1 <sup>a</sup>	177.8 <sup>b</sup>
Valeric	15.5 <sup>a</sup>	185.0 <sup>b</sup>
Isovaleric	25.4 <sup>b</sup>	162.1 <sup>a</sup>
Isobutyric	25.4 <sup>b</sup>	153.4 <sup>a</sup>
2-Methylbutyric	26.6 <sup>b</sup>	149.5 <sup>a</sup>
All four acids	26.4 <sup>b</sup>	153.2 <sup>a</sup>
SE (n = 3)	0.30	2.73

Data from Gorosito *et al.* (1985).

Each acid was added at 1.76 mM.

<sup>a,b</sup> Means within a column with different superscripts differ ( $P < 0.05$ ).

**Table 13.21** Effects of carbon-4 and carbon-5 fatty acids (straight or branched) on *in vitro* the plant cell wall digestion

Substrates
Alfalfa hay
Timothy hay
Wheat straw
Reed canary grass
Bermuda grass
Orchard grass
Corn silage
Filter paper <sup>c</sup>

<sup>a</sup> Expressed as percentage of

<sup>b</sup> No C<sub>4</sub> and C<sub>5</sub> acids were

<sup>c</sup> Each acid (valeric, isovaleric

<sup>d</sup> Means within a row differ

<sup>e</sup> Ball milled Whatman no.

Data from Gorosito *et al.* (

digestion, although v. digestibility (Mir *et al.* a isolated plant cell wal cell wall digestibility *al.*, 1985). Such posit branched-chain fatty 1985; Gorosito *et al.* served with all forage content (Table 13.21; lation was observed v significant response v tein content (Gorosi bacterial activity was *al.*, 1989) and bacteria *et al.*, 1987).

In contrast to the s on fiber digestibility or non-significant (A significant effect on rumen of beef heifer chain fatty acids. Sev mentation with isoac cows (Andries *et al.*, isoacids seems to in that, in four of five tr production by 4-8% trast, the response v

synthesis, has been  
mentations *in vitro*  
apolate the *in vitro*  
ence of a number of  
ity of microbes, and  
  
of minerals requires  
atory concentration  
erion, 1984; Durand  
ntration of one min-  
ie microbes and the

phenylacetic acids.  
utyric acid (2MBA)  
rly called 'isoacids',  
eral rumen bacterial  
1963; Bryant, 1973).  
mentation products  
ely (Allison, 1963).  
late fermentation.  
le of taking up the  
nthesize them from  
, 1967). Also, these  
ed, long-chain fatty  
a (Bryant, 1973).  
e importance of the  
on of branched-chain  
wn to stimulate fiber

or branched) on *in vitro*

nia concentration (mg l<sup>-1</sup>)

177.8 <sup>b</sup>
185.0 <sup>b</sup>
162.1 <sup>a</sup>
153.4 <sup>a</sup>
149.5 <sup>a</sup>
153.2 <sup>a</sup>
2.73

5).

**Table 13.21** Effects of carbon-4 (C<sub>4</sub>) and carbon-5 (C<sub>5</sub>) fatty acids (straight or branched) on the plant cell wall digestion from intact substrates<sup>a</sup>

Substrates	Control <sup>b</sup>	C <sub>4</sub> and C <sub>5</sub> acids <sup>c</sup>	Significance <sup>d</sup>
Alfalfa hay	34.1	38.7	*
Timothy hay	48.5	52.6	NS
Wheat straw	10.4	13.0	**
Reed canary grass	32.1	37.8	NS
Bermuda grass	18.2	21.8	NS
Orchard grass	30.2	38.0	*
Corn silage	34.1	36.7	*
Filter paper <sup>e</sup>	1.3	11.9	**

<sup>a</sup>Expressed as percentage dry matter.  
<sup>b</sup>No C<sub>4</sub> and C<sub>5</sub> acids were added.  
<sup>c</sup>Each acid (valeric, isovaleric, isobutyric and 2-methylbutyric) was added at 1.76mM.  
<sup>d</sup>Means within a row differ (\**P* < 0.05 or \*\**P* < 0.01); NS, not significant (*P* > 0.05).  
<sup>e</sup>Ball milled Whatman no. 1 filter paper.  
Data from Gorosito *et al.* (1985).

digestion, although valine alone had no effect in stimulating barley straw digestibility (Mir *et al.*, 1986). Incubations of mixed ruminal bacteria and isolated plant cell walls or intact forages with isoacids resulted in increased cell wall digestibility and ammonia assimilation (Table 13.20; Gorosito *et al.*, 1985). Such positive responses *in vitro* were obtained only with the branched-chain fatty acids and not with valerate (Cummins and Papas, 1985; Gorosito *et al.*, 1985). Also, the stimulatory response was not observed with all forage sources and apparently was not related to the protein content (Table 13.21; Gorosito *et al.*, 1985). For example, the highest stimulation was observed with filter paper, a pure cellulose substrate; however, a significant response was also obtained with alfalfa, a forage with high protein content (Gorosito *et al.*, 1985; Mir *et al.*, 1986). The stimulation of bacterial activity was also evidenced by increased VFA production (Kone *et al.*, 1989) and bacterial protein synthesis (Russell and Sniffen, 1984; Andries *et al.*, 1987).

In contrast to the studies *in vitro*, the results of supplementation *in vivo* on fiber digestibility and microbial protein production have been minimal or non-significant (Andries *et al.*, 1987). Gunter *et al.* (1990) observed no significant effect on fermentation and microbial protein synthesis in the rumen of beef heifers consuming grass hay supplemented with branched-chain fatty acids. Several investigators have studied the influence of supplementation with isoacids on the performance of growing cattle and dairy cows (Andries *et al.*, 1987). In high-yielding cows, supplementation with isoacids seems to increase milk production. Papas *et al.* (1984) reported that, in four of five trials, such supplementation increased the 305-day milk production by 4–8% in cows. Milk composition was not affected. In contrast, the response with growing cattle was minimal and suggested that

# The Rumen Microbial Ecosystem

*Edited by*

**P.N. HOBSON**

*Department of Molecular and Cell Biology  
University of Aberdeen*

*and*

**C.S. STEWART**

*The Nutrition Division  
The Rowett Research Institute  
Aberdeen*

**GOPHER, FTP OR EMAIL:**

A service of **ITP**<sup>®</sup>



**BLACKIE ACADEMIC & PROFESSIONAL**

*An Imprint of Chapman & Hall*

London · Weinheim · New York · Tokyo · Melbourne · Madras

Published by Blackie Academic & Professional, an imprint of  
Chapman & Hall, 2-6 Boundary Row, London SE1 8HN

Chapman & Hall, 2-6 Boundary Row, London SE1 8HN, UK

Chapman & Hall GmbH, Pappelallee 3, 69469 Weinheim, Germany

Chapman & Hall USA, 115 Fifth Avenue, New York, NY 10003, USA

Chapman & Hall Japan, ITP-Japan, Kyowa Building, 3F, 2-2-1  
Hirakawacho, Chiyoda-ku, Tokyo 102, Japan

DA Book (Aust.) Pty Ltd, 648 Whitehorse Road, Mitcham 3132, Victoria,  
Australia

Chapman & Hall India, R. Seshadri, 32 Second Main Road, CIT East,  
Madras 600 035, India

First edition 1988

Second edition 1997

© 1997 Chapman & Hall

Typeset in 10/12pt Times New Roman by Best-set Typesetter Ltd., Hong Kong  
Printed in Great Britain by St Edmundsbury Press, Bury St Edmunds, Suffolk

ISBN 0 7514 0366 0

Apart from any fair dealing for the purposes of research or private study,  
or criticism or review, as permitted under the UK Copyright Designs and  
Patents Act, 1988, this publication may not be reproduced, stored, or  
transmitted, in any form or by any means, without the prior permission in  
writing of the publishers, or in the case of reprographic reproduction only  
in accordance with the terms of the licences issued by the Copyright  
Licensing Agency in the UK, or in accordance with the terms of licences  
issued by the appropriate Reproduction Rights Organization outside the  
UK. Enquiries concerning reproduction outside the terms stated here  
should be sent to the publishers at the London address printed on this  
page.

The publisher makes no representation, express or implied, with regard  
to the accuracy of the information contained in this book and cannot  
accept any legal responsibility or liability for any errors or omissions that  
may be made.

A catalogue record for this book is available from the British Library

Library of Congress Catalog Card Number: 96-80242

Printed on acid-free text paper, manufactured in accordance with ANSI/  
NISO Z39.48-1992 (Permanence of Paper).

QR171  
R85  
R86  
1559

Dedicated to Bob

Applicant	: Cindie M. Luhman	
Serial No.	: 09/239,873	
Filed	: January 29, 1999	Group Art Unit: 1616
For	: METHOD AND COMPOSITION FOR ENHANCING MILK COMPONENT CONCENTRATIONS	Examiner: N. Levy
Docket No.	: LL11.12-0040	

# EXHIBIT I

of

## DECLARATION UNDER 35 U.S.C. § 132

Hobson, P.N., The Rumen Microbial Ecosystem,  
pages 140-141 (1988, 1<sup>st</sup> Edition, Elsevier Science Publishers Ltd.)



Methanogenic bacteria sometimes contaminate fresh isolates of rumen anaerobic fungi (Bauchop & Mountfort, 1981), but they can be removed by treatment with chloramphenicol.

When rumen fungi are cultured axenically on plant fibre, rhizoid tissue may form a mat on the surface of the fibre (Fig. 4). This is in contrast to fungi growing in the rumen where fungal rhizoids are located beneath the surface of the plant tissue.

### Culture media

The most commonly employed, undefined culture media used for growing anaerobic rumen fungi are similar to that of Orpin (1975), and consist of centrifuged rumen fluid, tryptone, yeast extract, a carbon source, a carbon dioxide-bicarbonate buffer at pH 6.7-6.9, L-cysteine as reducing agent, and vitamins. This medium may be solidified with 0.8-1.5% agar for use in isolating clones from single colonies (Joblin, 1981). If bacterial contamination of cultures occurs, antibiotics such as ampicillin, streptomycin and chloramphenicol may be incorporated into the medium.

Some strains of the polyflagellated species have been grown in defined media, for example *N. patriciarum* (Orpin & Greenwood, 1986b) and unidentified *Neocallimastix* spp. (Lowe *et al.*, 1985).

Culture media incorporating plant tissues as carbon source are valuable for ensuring that the organisms do not lose their ability to ferment plant structural carbohydrates *in vitro* (Orpin & Letcher, 1979). Cultures in the liquid media of Orpin (1975) gave high cell yields but needed subculturing after 24-48 hours to maintain viability, whereas cultures on plant tissues may be viable for up to 7 days, thus simplifying routine maintenance. Maintenance of cultures for periods of several months without subculturing has been achieved with *Neocallimastix* and *Piromonas* spp. by the storage at 39°C of cultures on plant tissues embedded in agar (Joblin, 1981). Long-term storage has been achieved by controlled freezing in liquid nitrogen or at -70°C using 5% dimethyl sulphoxide as cryoprotectant (Yarlett *et al.*, 1986b).

### Nutrition

Only one species, *Neocallimastix patriciarum*, has been grown in a minimal medium (Orpin & Greenwood, 1986b). Minimal nutritional requirements for growth of *N. patriciarum* on cellobiose in a CO<sub>2</sub> atmosphere were satisfied by the provision of sources of haem, D-biotin, thiamin or its precursors, ammonium ions, and of reduced sulphur and trace elements. Growth was stimulated by amino acids, straight and branched short-chain volatile fatty acids, low concentrations of long-chain fatty acids and a number of vitamins. Germination of zoospores was stimulated by acetic acid and soluble, fermentable carbohydrates.

Haems appear to play a major role in both the nutrition and zoosporogenesis of rumen fungi (Orpin, 1986a). Although haems probably occur in rumen contents during the entire day, the addition of haems to rumen contents of animals fed once per day resulted in zoosporogenesis of *Neocallimastix* sp., but this could not be satisfactorily repeated *in vitro* with pure cultures, suggesting that the control of

zoosporogenesis work *in vivo*. It stimulates zoospore haems in an oxygen-reduced rather than monoflagellate

In *Neocallimastix* major glycolytic pathways (1986a). This is a fermenting glucose. C. G., unpublished.

Glycolysis gives rise to malate. It is malate that is pyruvate, pyruvate, and pyruvate, and the pyruvate is oxidoreductase responsible for the conversion of acetaldehyde into hydrogenosomes by end-product decarboxylase. It was found to require 100% N<sub>2</sub> atmosphere.

It is clear that those that can digest cellulose (Bauchop, 1985) hydrolyse a range of carbohydrates (Asher, 1983). Many vegetative stages of extracellular and particular active *frontalis* and *N. communis* and

zoosporogenesis and zoospore release may be more complex than suggested by the work *in vivo*. Haems may act synergistically with other dietary components to stimulate zoosporogenesis. Because of the low redox potential of the rumen, dietary haems in an oxidised state would be rapidly reduced, and it is likely that the reduced rather than the oxidised haem is functional *in vivo*. Zoosporogenesis in monoflagellated species may also be triggered by similar compounds (Orpin, 1977*d*).

### INTERMEDIARY METABOLISM

In *Neocallimastix patriciarum* the Embden-Meyerhof pathway appears to be the major glycolytic pathway used in the catabolism of carbohydrates (Yarlett *et al.*, 1986*a*). This is supported by the distribution of  $^{14}\text{CO}_2$  produced by cultures fermenting glucose which had been labelled in C-1, C-2 and C-6 positions (Orpin, C. G., unpublished).

Glycolysis generates phosphoenolpyruvate which is converted via oxaloacetate into malate. Hydrogenosomal enzymes generate energy from the oxidation of malate to pyruvate which is coupled, via NADPH:ferridoxin oxidoreductase, ferridoxin and hydrogenase, to production of molecular hydrogen. Some of the pyruvate generated is converted into acetyl-CoA via pyruvate:ferridoxin oxidoreductase, and subsequently into acetate. Cytosolic lactate dehydrogenase is responsible for lactate generation and, when cultures are grown under a  $\text{N}_2$  rather than under a  $\text{CO}_2$  atmosphere, low levels of alcohol dehydrogenase convert acetaldehyde into ethanol (Yarlett *et al.*, 1986*a*). Carbon dioxide appears to suppress hydrogenosomal acetate generation as well as cytosolic ethanol production, perhaps by end-product inhibition of the pyruvate:ferridoxin oxidoreductase and pyruvate decarboxylase reactions, both of which generate  $\text{CO}_2$ . One strain of *Neocallimastix* was found to require 7%  $\text{CO}_2$  in the gas phase and could not be grown under a 100%  $\text{N}_2$  atmosphere (Joblin, K., unpublished).

### FERMENTATION OF CARBOHYDRATES

It is clear that the rumen fungi so far examined produce a wide range of enzymes that can digest the major structural carbohydrates of plant cell walls (Pearce & Bauchop, 1985; Wood *et al.*, 1986; Williams & Orpin, 1987*a*; Lowe *et al.*, 1987*b,c*), hydrolyse a range of glycosidic linkages (Williams & Orpin, 1987*b*), and enable the fungi to grow on a number of polysaccharides (Orpin & Letcher, 1979; Mountfort & Asher, 1983). Many of the polysaccharide-hydrolysing enzymes are produced by the vegetative stage and the zoospores of the fungi, and are also present in the extracellular culture medium (Williams & Orpin, 1987*a,b*; Lowe *et al.*, 1987*b*). The particular activity depends upon the growth substrate. Thus *Neocallimastix frontalis* and *N. patriciarum* will utilise cellulose, xylan, starch and hemicelluloses (Orpin & Letcher, 1979; Bauchop, 1983; Orpin & Munn, 1986), as will *P. communis* and another monoflagellated species (Williams & Orpin, 1987*a*). No

# THE RUMEN MICROBIAL ECOSYSTEM

*Edited by*

**P. N. HOBSON**

*Honorary Research Fellow, Biochemistry Department,  
Marischal College, Aberdeen University,  
Aberdeen, UK*

*Formerly Head, Microbial Chemistry Department,  
Rowett Research Institute, Aberdeen, UK*



**ELSEVIER APPLIED SCIENCE**  
LONDON and NEW YORK

QK  
171  
.P85

ELSEVIER SCIENCE PUBLISHERS LTD  
Crown House, Linton Road, Barking, Essex IG11 8JU, England

*Sole Distributor in the USA and Canada*  
ELSEVIER SCIENCE PUBLISHING CO., INC.  
52 Vanderbilt Avenue, New York, NY 10017, USA

WITH 68 TABLES AND 48 ILLUSTRATIONS

© 1988 ELSEVIER SCIENCE PUBLISHERS  
(except Chapter 10: © Government of Canada)

**British Library Cataloguing in Publication Data**

The Rumen microbial ecosystem.  
1. Ruminants. Rumen. Microflora  
I. Hobson, P.N.  
599.73'504132

**Library of Congress Cataloging in Publication Data**

The Rumen microbial ecosystem.  
1. Rumen—Microbiology. I. Hobson, P. N.  
QR171.R85R86 1988 599.73'504132 88-16424

ISBN 1-85166-188-3

No responsibility is assumed by the Publisher for any injury and/or damage to persons or property as a matter of products liability, negligence or otherwise, or from any use or operation of any methods, products, instructions or ideas contained in the material herein.

**Special regulations for readers in the USA**

This publication has been registered with the Copyright Clearance Center Inc. (CCC), Salem, Massachusetts. Information can be obtained from the CCC about conditions under which photocopies of parts of this publication may be made in the USA. All other copyright questions, including photocopying outside the USA, should be referred to the publisher.

All rights reserved. No part of this publication may be reproduced, stored in a retrieval system, or transmitted in any form or by any means, electronic, mechanical, photocopying, recording, or otherwise, without the prior written permission of the publisher.

Printed in Great Britain by Galliard (Printers) Ltd, Great Yarmouth

QR  
171  
R85  
R86  
1988

Ruminants have and have provided kinds of climates vegetation from

The ability to rumen, and this was known 100 c not until 40 or 5 digestion of vega

In the late 194 nutrition of the said to have a d

Progress was conversion of v bacteria and pro or investigated in in the rumen an reactions were kn rumen microbiol *The Rumen and*

Since then, pu with other scienti but new techni microorganisms, non-ruminant I demonstrated.

The rumen is a many reactions. E habitats other the

Applicant	: Cindie M. Luhman	
Serial No.	: 09/239,873	
Filed	: January 29, 1999	Group Art Unit: 1616
For	: METHOD AND COMPOSITION FOR ENHANCING MILK COMPONENT CONCENTRATIONS	Examiner: N. Levy
Docket No.	: LL11.12-0040	

# EXHIBIT J

of

## DECLARATION UNDER 35 U.S.C. § 132

Santoro, Luiz G., Grant, George, and Pusztai, A. Arpad, Differences in the Degradation In Vivo and In Vitro of Phaseolin, the Major Storage Protein of *Phaseolus Vulgaris* Seeds, pages 612-613, Biological Society Transactions - 625<sup>th</sup> meeting, (London 1998)

phonic analogue is not recognized. These collected findings are consistent with recent crystallographic and chemical studies on the active site of the enzyme (Sandmeier & Christen, 1982; Arnone *et al.*, 1984; Kirsch *et al.*, 1984): the substrate specificity is largely determined by the interactions of  $\alpha$ - and  $\omega$ -carboxylate groups with guanidinium groups of Arg-386 and Arg-292, respectively. The binding of the two carboxylate groups induces a conformational change of the protein, and the  $\epsilon$ -amino group of Lys-258 which binds the coenzyme can act as  $C_{\alpha}$ -H proton acceptor. It may be assumed that the replacement of one of the carboxylate groups induces restrictions in such conformational changes, leading to a decrease in reactivity of the effector. Distinctions in cytosolic and mitochondrial isozymes with respect to the distances between the two arginyl residues and the lysyl 258 Schiff's base, and/or the degree of freedom of the rotation of these active site components (Iriarte *et al.*, 1984) could explain the different data observed for Asp- $\beta$ -P and Glu- $\gamma$ -P with the two isozymes.

The present results relative to the aminomalonate analogue and Asp- $\beta$ -P, which are both substrates and inhibitors, show the importance of an  $\alpha$ -carboxylic group in the binding of an effector with the ASAT active site.

Arnone, A., Rogers, P. H., Hyde, C. C., Makinen, M. W., Feldhaus, R., Metzler, C. M. & Metzler, D. E. (1984) in *Chemical and Biological Aspects of Vitamin B6 Catalysis* (Evangelopoulos, A. E., ed.), part B, pp. 171-193, Alan R. Liss, Inc., New York

Brand, L. M. & Lowenstein, J. M. (1978) *Biochemistry* 17, 1365-1370  
 Cheng, S., Michuda-Kozak, C. & Martinez-Carrion, M. (1971) *J. Biol. Chem.* 246, 3623-3630  
 Christen, P. & Metzler, D. E. (eds.) (1985) *Transaminases*, John Wiley & Sons, New York  
 Dolphin, D., Poulson, R. & Avramovic, O. (eds.) (1986) *Vitamin B6 Pyridoxal Phosphate*, part B, John Wiley & Sons, New York  
 Heller, M. & Gehring, H. (1984) in *Chemical and Biological Aspects of Vitamin B6 Catalysis* (Evangelopoulos, A. E., ed.), part B, pp. 153-159, Alan R. Liss, Inc., New York  
 Iriarte, A., Relimpio, A. M., Chlebowski, J. F. & Martinez-Carrion, M. (1984) in *Chemical and Biological Aspects of Vitamin B6 Catalysis* (Evangelopoulos, A. E., ed.), part B, pp. 107-115, Alan R. Liss, Inc., New York  
 Kirsch, J. F., Eichele, G., Ford, G. C., Vincent, M. G., Janssonius, J. N., Gehring, H. & Christen, P. (1984) *J. Mol. Biol.* 174, 497-525  
 Lejczak, B., Starzemska, H. & Mastalerz, P. (1981) *Experientia* 37, 461-462  
 Recasens, M., Benezra, R., Basset, P. & Mandel, P. (1980) *Biochemistry* 19, 4583-4589  
 Sandmeier, E. & Christen, P. (1982) *J. Biol. Chem.* 257, 6745-6750  
 Walter, U., Luthe, H., Gerhart, F. & Söling, H. D. (1975) *Eur. J. Biochem.* 59, 395-403  
 Yagi, T., Kagamiyama, H. & Nozaki, M. (1979) *Biochem. Biophys. Res. Commun.* 90, 447-452

Received 23 November 1987

## Differences in the degradation *in vivo* and *in vitro* of phaseolin, the major storage protein of *Phaseolus vulgaris* seeds

LUIZ G. SANTORO, GEORGE GRANT and ARPAD PUSZTAI

Biochemistry Division, Rowett Research Institute, Greenburn Road, Bucksburn, Aberdeen AB2 9SB, U.K.

Storage proteins of legume seeds are considered to be poorly digestible in their native form (Liener & Thompson, 1980). The partial resistance of native phaseolin (glycoprotein II, G-II), the main storage protein of *Phaseolus vulgaris*, to degradation by pepsin, trypsin and chymotrypsin, *in vitro*, alone or in a sequential combination (Liener & Thompson, 1980; Deshpande & Nielsen, 1987) has been demonstrated. Limited proteolysis by trypsin or chymotrypsin reduced the size of the native protein from 140 kDa to 120 kDa (Liener & Thompson, 1980). The subunits of G-II were cleaved near the middle of the polypeptide chains and the products in SDS had  $M_r$  values in the range of 22-30 kDa. According to Vaintraub *et al.* (1976) and R. Begbie (unpublished work), the hydrolysis *in vitro* of undenatured G-II by pepsin resulted in only about 2% of the total peptide bonds being broken. On the other hand, G-II appeared to be more extensively degraded *in vitro*, if instead of pure gut endopeptidases, stomach and small intestinal luminal and tissue extracts were used as sources of proteolytic enzymes (Sgarbieri *et al.*, 1982).

As there is much less information available about the degradation *in vivo* of pure G-II, in the present work, the true digestibility *in vivo* of a highly purified G-II preparation was studied in rats.

G-II was isolated from seeds of *P. vulgaris* as before (Pusztai & Watt, 1970). The amount of lectin contamination was reduced to less than 0.3% by affinity chroma-

tography on Sepharose 4B-fetuin. It was thought unlikely that the results would be influenced unduly by the presence of such a small amount of toxic component.

Rats fasted for 16 h were given an intragastric dose of 300 mg of G-II (45 mg N) and then fed a protein-free diet, *ad lib*, for 3 days. Faeces were collected daily and N estimated. Faeces were also extracted with 0.025 M-glycine/Tris buffer, pH 8.6 (3-5 mg of faeces/ml of buffer) and the concentration of G-II-related material was estimated by rocket immunoelectrophoresis. This was based upon previous observation (R. Begbie, unpublished work) that the fragment derived from the partial digestion of G-II retained full reactivity with anti-G-II antibodies.

It was found that (Fig. 1a) the faeces contained more than 50 mg of total N although only 45 mg of G-II N had been introduced into the stomach. The faecal N comprised approx. 12 mg of G-II N. The remainder, about 38 mg, was unrelated to G-II, indicating that 74% of the G-II was degraded during passage through gastrointestinal tract. The bulk (8 mg) of the partially digested G-II N appeared in the faeces in the first 24 h, while the output of N unrelated to G-II peaked on the second day. It appears that by an unknown mechanism, the native G-II and/or its fragments may have stimulated an increased secretion of endogenous metabolic N.

Since proteolysis by bacteria in the large intestine may have made appreciable contributions to degradation of G-II without nutritionally benefiting the rat, in another series of experiments rats were given an intragastric dose of 150 mg of G-II (22.5 mg N) and killed after 1 h. Both stomach and small intestine were removed and their contents washed out. The tissues were then homogenized in phosphate buffer containing aprotinin (5 mg tissue/ml of buffer) and centrifuged. After correction for the control (rats given an intragastric

Abbreviation used: G-II, glycoprotein II.

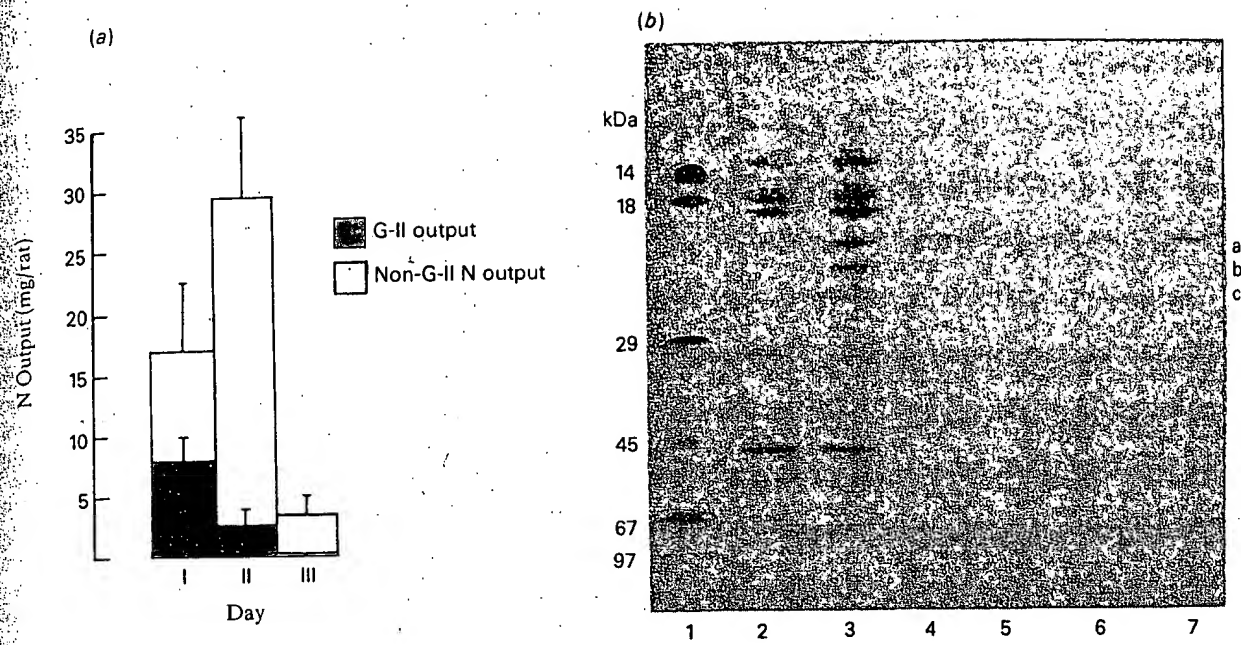


Fig. 1. (a) Total N (corrected for normal metabolic faecal N) and G-II N output over 3 days in the faeces of rats given 300 mg of G-II (45 mg N) and (b) SDS/PAGE of G-II related polypeptides

Lane 1, standards; lane 2, extract from the small intestine tissue of control rats; lane 3, extract from the small intestine tissue of rats given an intragastric dose of G-II; lane 4, G-II core-polypeptides recovered in luminal contents of rats given G-II; lane 5, native G-II; lanes 6 and 7, G-II core-polypeptides from faeces; (a)  $\approx$  22 kDa, (b)  $\approx$  23 kDa, (c)  $\approx$  25 kDa.

dose of saline), the total amount of N found in the luminal contents and tissue homogenates was at least as much ( $23.4 \pm 0.8$  mg) as in the G-II input. On the other hand, the amount of G-II-related N recovered from the small intestine was only  $9.6 \pm 0.8$  mg, indicating that about 57% of the G-II was degraded and absorbed within 1 h. Moreover, a large proportion (about 60%) of the surviving G-II-related protein was found to be strongly associated with small intestinal tissue (Fig. 1b) and was released only on homogenization.

In conclusion, at least 57% of G-II was degraded in the small intestine within 1 h. During the remaining time ( $\approx$  1 h) in the small intestine and then in the large intestine, G-II was further degraded. The core-polypeptide fragments of 22–30 kDa surviving in the small intestine showed a similar subunit pattern by SDS/polyacrylamide gel electrophoresis (PAGE) to that observed on degradation *in vitro* (Deshpande & Nielsen, 1987). As the extent of degradation of G-II *in vivo* (74%) exceeds that obtained *in vitro* by pure endopeptidases (2%), the results strongly suggest the involvement of additional proteinases in the breakdown of G-II in the gut. The strong attachment of G-II to the intestinal tissue and extended exposure time to proteolytic enzymes may further aid this digestive process. However, an appreciable part (about 26%) of the dietary G-II escapes digestion in the whole alimentary tract. The reasons for this may be related

to the previously observed microheterogeneity of G-II (Pusztai & Stewart, 1980) or partial protection from proteolysis by an unknown mechanism.

Finally the large difference between apparent and true digestibility values indicates that G-II and/or its fragments are stimulants of secretion of endogenous N (mucus, etc.) in the small intestine.

L. G. Santoro gratefully acknowledges the receipt of a post-graduate scholarship from E.M.B.R.A.P.A. (Brasil).

Deshpande, S. S. & Nielsen, S. S. (1987) *J. Food Sci.* **52**, 1326–1329  
Liener, I. E. & Thompson, R. M. (1980) *Qual. Plant. Plant Foods Hum. Nutr.* **30**, 13–25  
Pusztai, A. & Stewart, J. C. (1980) *Biochim. Biophys. Acta* **623**, 418–428  
Pusztai, A. & Watt, W. B. (1970) *Biochim. Biophys. Acta* **207**, 413–431  
Sgarbieri, V. C., Clarke, E. M. W. & Pusztai, A. (1982) *J. Sci. Food Agric.* **33**, 881–891  
Vaintraub, I. A., Bassuner, R. & Shutov, A. D. (1976) *Nahrung* **20**, 763–771

Received 2 December 1987

Applicant	: Cindie M. Luhman	
Serial No.	: 09/239,873	
Filed	: January 29, 1999	Group Art Unit: 1616
For	: METHOD AND COMPOSITION FOR ENHANCING MILK COMPONENT CONCENTRATIONS	Examiner: N. Levy
Docket No.	: LL11.12-0040	

EXHIBIT K

of

DECLARATION UNDER 35 U.S.C. § 132

**U.S. Patent No. 4,127,676 to Merensalmi**



# United States Patent [19]

Merensalmi

[11] 4,127,676

[45] Nov. 28, 1978

[54] FODDER ADDITIVE FOR RUMINANTS

[75] Inventor: Matti J. Merensalmi, Turku, Finland

[73] Assignee: Farnos-Yhtymä Oy, Finland

[21] Appl. No.: 778,359

[22] Filed: Mar. 17, 1977

[30] Foreign Application Priority Data

Mar. 19, 1976 [FI] Finland ..... 760746

[51] Int. Cl.<sup>2</sup> ..... A23K 1/00; A23K 1/02

[52] U.S. Cl. .... 426/2; 426/635;  
426/640; 426/807

[58] Field of Search ..... 426/635, 807, 2, 640,  
426/636; 260/635 C

[56] References Cited

## FOREIGN PATENT DOCUMENTS

600,707 6/1960 Canada ..... 426/807  
645,019 7/1962 Canada ..... 260/635 C  
838,766 6/1960 United Kingdom ..... 260/635 C

Primary Examiner—Raymond N. Jones

Assistant Examiner—Elizabeth A. Hatcher

Attorney, Agent, or Firm—McGlew and Tuttle

[57]

## ABSTRACT

A fodder additive is provided for increasing the blood sugar of ruminants and their milk level. The fodder additive comprises as active agents at least one sugar alcohol having five or six hydroxyl groups, especially xylitol, arabitol and dulcitol. A preferred additive contains, based upon dry weight: 5-25% xylitol; 20-35% arabitol; 10-25% mannitol; 5-15% sorbitol; 5-10% dulcitol; 5-10% rhamnitol.

9 Claims, No Drawings

## FODDER ADDITIVE FOR RUMINANTS

### BACKGROUND AND OBJECT OF THE INVENTION

The object of the invention is a fodder additive for use in the feeding of ruminants, the additive influencing advantageously the milk production of cows by increasing their blood sugar level.

The metabolism of the organism requires easily soluble carbohydrates, above all various sugars, of which glucose is the most important. In the non ruminants the large molecular carbohydrates, such as starch, split into sugars by means of the digestive fluid in the intestine. In ruminants, the same splitting takes place already in the proventriculi, above all in the rumen, through the action of microbes. These carbohydrates as well as the sugars contained in the fodder are used as an energy source of the microbes and are thus converted into a microbial mass and consequently do not directly increase the sugar supply to the organism.

Animals, including ruminants, need sugar predominantly for the function of the liver and the mammary glands. Glucose is converted into lactose in the cow's udder and is secreted together with the milk. In a cow yielding 30 kg of milk daily this corresponds to about 1500 g of glucose leaving the organism daily. Taking into consideration the animal's further requirements it is estimated that a medium size cow in its period of maximum production requires about 2000 g/day of sugar. This sugar amount must be supplied by the liver. As no considerable amounts of sugar, especially glucose, pass through the rumen, intact glucose must be produced in the liver of the ruminants. Important glucose sources constitute propionic acid in the rumen and glucose synthesis by the organism from proteins and lactic acid. It is however estimated that the sugar deficit in an animal yielding 30 kg of milk daily is about 700 g. The animal attempts to compensate for this deficit by breaking down organism fats. By this means glycerol is formed which is converted into glucose. Also fatty acids are formed, which in turn break down into ketone-bodies. Thus, the animal might suffer from ketosis in case an excessive degradation of fats occurs.

If the supply of glucose to the animal is ensured, the breaking down of fat tissue decreases which, however, is not normally sufficient to maintain the glucose level in the blood.

The glucose level in the udder plays an essential role in the regulation of the milk yield. If the udder tissue contains large amounts of glucose, the cells will absorb fluid by osmosis from the blood to compensate the osmotic pressure. The milk yield thus increases. If the sugar content is low, the opposite phenomenon occurs.

### THE PRIOR ART

As today's high yield ruminants are not always capable of ensuring their glucose need, auxiliary feeding means have been used. Ruminants have been administered glucogenic feeding additives which do not break down in the rumen, but pass intact therethrough to the later stomach compartments and from there on to the liver, where they are converted into sugars. It is also possible to try to increase the propionic acid content in the rumen as propionic acid is converted into active glucose in the liver. For this purpose it is known to use the following substances: propylene glycol, glycerol, various propionates, even propionic acid.

## SUMMARY OF THE INVENTION

According to the invention it has now surprisingly been found that an additive to be incorporated into ruminant fodder and exhibiting a high sugar alcohol content, an advantageous composition of which is found in the mixture of various sugar alcohols obtained as a by-product in the production of xylitol from plant material, substantially improves the glucose balance of ruminants as it acts similarly to the above mentioned glucogenic substances. The characteristics of the additive appear in the appended claims.

An advantageous composition contains xylitol, arabitol, dulcitol and possibly rhamnitol.

### DESCRIPTION OF EMBODIMENTS OF THE INVENTION

The product according to the invention is called a sugar alcohol mixture, one embodiment of which is obtained as a by-product of the xylitol production from birch. It is a yellowish brown sweet fluid exhibiting a specific weight of about 1.22 as a 50 percent aqueous solution at room temperature. Its energy (caloric) value and degree of sweetness are same as for glucose. A composition of the product in question was earlier approximately the following, calculated on a dry matter basis:

xylitol	15-25 %	by weight
arabitol	20-35 %	"
mannitol	15-25 %	"
sorbitol	5-15 %	"
dulcitol	5-15 %	"
rhamnitol	5-10 %	"
others	2-5 %	"
break-down products	2-5 %	"

In more recent processes the recovery of xylitol has been improved and the composition may vary within the following limits:

xylitol	6-18 %	by weight
arabitol	9-21 %	"
mannitol	13-19 %	"
sorbitol	8-12 %	"
dulcitol	5-11 %	"
rhamnitol	4-6 %	"
reducing sugars	8-15 %	"
other polyols	6-14 %	"

It is known from literature that xylitol stimulates the liver functions and enhances the cell activity. It is also known that the microbes of the mouth are not able to utilize xylitol as an energy source. The present invention, however, is based upon the prior unknown fact that sugar alcohols remain intact also under the conditions in the rumen sufficiently long without breaking down, which appears from the test results presented in Example 1.

### EXAMPLE 1

In Example 1 the breaking down of various single sugar alcohols in the rumen has been studied in vitro.

The preservation of sugar alcohols in the rumen fluid (% of the amount added)

Incubation time	2h	4h	8h	24h	48h
xylitol	91.2	89.7	88.4	83.4	33.8
arabitol	86.8	93.0	88.3	87.0	55.9
mannitol	88.9	88.1	80.5	1.4	0.4

## -continued

Incubation time	2h	4h	8h	24h	48h
dulcitol	92.9	94.3	85.8	61.2	7.0
sorbitol	91.3	88.3	79.6	11.1	0.2

It is concluded that the sugar alcohols in question remain almost completely intact without being metabolized for 8 hours, xylitol, arabitol and dulcitol even for 24 hours and arabitol and xylitol partly even longer. Ordinary sugar breaks down in less than 2 hours.

## EXAMPLE 2

This Example concerns the behaviour in the rumen fluid of a sugar alcohol mixture of the following composition calculated as dry matter

xylitol	18 %	by weight
arabitol	24 %	"
mannitol	18 %	"
sorbitol	9 %	"
galaxitol	7 %	"
rhammitol	7 %	"
reducing sugars	7 %	"
other polyols	10 %	"

and it is concluded that its preservation is comparable to the preservation of the single sugar alcohols. Incubation time 2h 4h 8h 24h 48h % left of the amount added 92.6 86.7 87.6 55.2 23.7

As the flow of the fluid in the rumen is only a few hours, the sugar alcohol reaches the latter stomach compartments before any essential degradation in the rumen can occur.

After passing through the rumen the sugar alcohols act according to the normal sugar metabolism, which is evidenced i.a. as an increase in the blood glucose level as shown in Example 3.

## EXAMPLE 3

## Changes in the blood glucose level

days after start of test	Test period										
	0	10	13	15	17	18	19	21	25	Sugar alcohol	
No sugar alcohol											
glucose mmol/l	3.4	3.1	3.0	2.5	2.7	3.1	3.2	3.2	3.5	2 x 125 g dry matter	2 x 250 g dry matter

During normal feeding the glucose level of the test animal decreased constantly, and was less than 3.0 mmol/l already before starting the test feeding. The administration of 2 x 125 g dry matter of the sugar alcohol mixture according to Example 2 was begun on the 17th day of the test period. The blood glucose values showed an increase the very same day. After one week of test feeding the glucose value re-established its initial value at the beginning of the test period. The animal's general condition returned to normal, as judged externally.

The fat percentage in the milk of the test animal varied before administration of the sugar alcohol mixture by almost 2 percentage units daily, being at an average 4.5%. After starting the test feeding the fat percentage decreased within two days to the level specific for the

animal in question, i.e. below 4.0%. Simultaneously the daily fluctuations became insignificant.

The energy and above all the sugar metabolism of the animal of Example 3 was disturbed to such a degree that ketose would have been imminent had not the test feeding been started and the sugar metabolism restored to normal.

By acting on the rumen functions by increasing the propionic acid production at the expense of acetic acid and butyric acid, the sugar metabolism of ruminants may be improved, as propionic acid is converted to extremely effective glucose in the organism.

Example 4 illustrates the effect of a sugar alcohol mixture on the fatty acid production of the rumen. An increase in the acetic acid content means an energy waste, as its further metabolization gives rise to carbon dioxide which leaves the organism unused. On the other hand, propionic acid is converted entirely into glucose.

## EXAMPLE 4

Rumen specimens obtained with a fistula were investigated as to the changes in the acetic acid (E) and propionic acid (Pr) contents of the rumen fluid. During the pre-feeding stage the percentage of E showed an increase whereas that of Pr decreased. After starting the test feeding the ratio began to change into the opposite direction. Externally judged, the animal visibly improved.

Test period	1	2	3	4	5	6
E %	51	60	62	57	51	52
Pr %	28	26	25	27	28	29

Test stages 1 to 3 correspond to winter feeding with standard fodder, whereby symptoms of imbalance in the sugar metabolism were evident during feeding, at stage 4 the cow was fed a sugar alcohol mixture the composition of which corresponded to Example 2, in an amount of 2 x 125 g, at stage 5 the dose was 2 x 250 g and at stage 6 the dose was 2 x 200 g of sugar alcohols daily calculated as dry matter per animal.

It is concluded that the propionic acid formation by the rumen increased by more than 100 g daily as a result of the sugar alcohol mixture. The major part of the sugar alcohol mixture however passes through the rumen without breaking down.

## EXAMPLE 5

A cow that had already passed its high-yielding stage after calving was administered a solution containing sugar alcohols (c.f Example 2) at an amount of 0.4 liters daily. The composition of the solution was:

propylene glycol	10 %	by weight
sodium propionate	5 %	"
sugar alcohol mixture (50 % dry matter)	40 %	"
molasses (60 % dry matter)	45 %	"
	100 %	(~ 65 % dry matter)

The milk yield during the test feeding which lasted for about 4 weeks increased clearly, the increase being 0.1 kg daily. The test animal had already passed its peak yield at the beginning of the test feeding, the energy requirement as glucose thus having already decreased from its maximum value. Despite this the animal increased its milk production. A similar test cannot be

5

carried out during the early lactation, as it is not possible to say how much of the production increase is due to the normal increasing of the yield and how much is due to the fodder additive.

As a vehicle or fodder component for the additive mixture e.g. a by or waste product of a nutrient production process, such as molasses, is advantageously used. Into the mixture may also be incorporated glucogenic substances known per se, such as propylene glycol, whereby the amount of sugar alcohol mixture may be correspondingly reduced. The amount of sugar alcohol mixture is, however, always at least 10% by weight calculated on the dry matter of the mixture.

The additive mixture according to the invention may be used as a solution, whereby it may be packed in suitable containers and administered directly during feeding. The recommended dosage is 40 to 500 g of sugar alcohol daily calculated on the dry matter, per animal. The additive may also be dried and added e.g. to other industrial fodders, or used as such as dry bulk feeds.

I claim:

1. Method of increasing milk yield in a ruminant which comprises enriching fodder for said ruminant with an additive consisting essentially of a sugar alcohol having five or six hydroxyl groups to the fodder of the ruminant in an amount of at least 10% by weight calcu-

6

lated on the dry matter of fodder and feeding said enriched fodder to the ruminant in an amount sufficient to administer 40 to 500 grams daily of said sugar alcohol to said ruminant, whereby the milk yield from said ruminant is increased.

2. Method according to claim 1, wherein said additive contains a member selected from the group consisting of xylitol, arabitol, dulcitol and mixtures thereof.

3. Method according to claim 1, wherein the sugar alcohols comprise by-products obtained from the xylitol production from plant material.

4. Method according to claim 3, wherein the additive contains, calculated on the weight of dry matter, xylitol 5 to 25% arabitol 20 to 35%, mannitol 10 to 25%, sorbitol 5 to 15% dulcitol 5 to 10%, and rhamnitol 5 to 10%.

5. Method according to claim 1, wherein the fodder comprises a by-product from a nutrient production process, such as molasses.

6. Method according to claim 1, wherein said additive further contains a glucogenic substance.

7. Method according to claim 6, wherein said glucogenic substance is propylene glycol.

8. Method according to claim 1, wherein said additive is in the form of a solution.

9. Method according to claim 1, wherein said additive is in the dried form.

\* \* \* \* \*

30

35

40

45

50

55

60

65